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**PROCEEDINGS OF THE 7TH ANNUAL CONFERENCE ON
ENVIRONMENTAL TOXICOLOGY
13, 14 and 15 OCTOBER 1976**

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TECHNICAL REVIEW AND APPROVAL

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The experiments reported herein were conducted according to the "Guide for the Care and Use of Laboratory Animals," Institute of Laboratory Animal Resources, National Research Council.

This report has been reviewed by the Information Office (OI) and is releasable to the National Technical Information Service (NTIS). At NTIS, it will be available to the general public, including foreign nations.

This technical report has been reviewed and is approved for publication.

FOR THE COMMANDER


ANTHONY A. THOMAS, MD
Director
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Inhalation toxicology	Pathology							
Environmental toxicology	Environmental Carcinogenesis							
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PREFACE

The Seventh Conference on Environmental Toxicology was held in Dayton, Ohio on 13, 14, and 15 October 1976. Sponsor was the University of California, Irvine under the terms of Contract F33615-76-C-5005 with the Aerospace Medical Research Laboratory, Aerospace Medical Division, Air Force Systems Command, Wright-Patterson Air Force Base, Ohio. Arrangements were made by the Toxic Hazards Research Unit of the University of California, Irvine, and the papers presented at this Conference by personnel of the University of California represent research conducted under the cited contract. Seymour L. Friess, Ph.D., Naval Medical Research Institute, National Naval Medical Center, Bethesda, Maryland served as Conference Chairman, and Mrs. Lois Doncaster, University of California, served as Conference Coordinator.

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OPENING ADDRESS

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Welcome to the Seventh Conference on Environmental Toxicology. In looking over the program for this Conference I have noticed a significant increase in active participation from a wide variety of universities and teaching institutions. These new contributors include such famous institutions as the University of Montreal, the University of Washington, The New York University Medical Center, the City of Hope Medical Center, the Harvard School of Public Health, the University of Pittsburgh Graduate School of Public Health, and the Naylor Dana Institute of the American Health Foundation.

The University of California and all the session chairmen certainly deserve great credit for organizing a very interesting and diverse scientific program for the next 2½ days. I also note the very welcome participation from the United States Army which gives us the opportunity to hear about their activities in toxicology and environmental research and development. We are particularly happy to see Dr. Friess from the U. S. Naval Medical Research Institute chairing this conference. This gives me the opportunity to personally welcome, in the name of the Commander of the Aerospace Medical Division, the toxicology detachment from Dr. Friess' Institute to the Aerospace Medical Research Laboratory at Wright-Patterson Air Force Base. At breakfast this morning, Dr. Friess and I were discussing this and I understand that you're going to have initially 4 or 5 people here, permanently assigned working in the Aerospace Medical Research Laboratory and that number will eventually increase to close to a dozen. We are most pleased to see this degree of cooperation.

Under a Memorandum of Agreement between the AMD and the Naval Medical Research and Development Command, the normobaric toxicology program of the former Navy Toxicology Unit will have access to the unique inhalation exposure facilities of the Toxic Hazards Division. We have entered into this agreement with a

spirit of mutual cooperation and support and with the objective of increasing the effectiveness of our respective programs in toxicology and related disciplines. We are convinced that this will be a great advantage to both of our services.

And finally, I would like to take this opportunity to introduce the new Commander of the Aerospace Medical Research Laboratory, Dr. Roy DeHart. Col DeHart has been here only about 3 months. Many of you who have been here before will, of course, remember Col Fred Doppelt who departed 3 months ago. Col Doppelt is now the Commander of the Air Force Hospital at Davis Monthan Air Force Base in Arizona.

With that, General Unger and I (and I'm sure I speak for Dr. DeHart), wish you a very successful and a profitable conference with a free exchange of ideas.

INTRODUCTORY REMARKS

Seymour L. Friess, Ph.D.

Naval Medical Research Institute
Bethesda, Maryland

I should like to take this opportunity to greet you on behalf of the Navy as a personal representative of the Commanding Officer of the Naval Medical Research Institute, Captain K. W. Sell. Dr. Sell has asked that I tell you about the motivation behind the Navy decision to join with the Air Force and the University of California on utilization of the splendid toxicology inhalation facility at the Toxic Hazards Research Unit, Wright-Patterson Air Force Base.

Essentially, in 1975 the Navy reached the assessment that it was falling short in meeting its responsibilities to the fleet for provision of inhalation toxicity data to be used in setting Permissible Exposure Limits and Emergency Exposure Limits. The shortfall was such as to require an immediate increase in data production by nearly a factor of two. Therefore, after a series of discussions between Navy and Air Force personnel, a policy decision was made to augment Navy data production in inhalation toxicology by making joint use of the THRU facility in a very intensive program involving continuous, long-term exposures.

It may also be of some interest to you to scan an overview of the administrative structure through which the Navy Medical Department incorporates its new toxicological information into the limit setting process. The heart of the structure (Figure 1) lies in the command element, the Naval Medical Research and Development Command (NMRDC), which directs and funds all toxicological research in the Navy. In turn, all data and results return to the NMRDC for preliminary evaluation before transmission to the Occupational Safety and Health Branch (Code 55) of the Bureau of Medicine and Surgery for promulgation of safe exposure limits for man. In part, the promulgation of exposure limits for fleet use involves an advisory function obtained through contract with the Committee on Toxicology of the National Research Council.

U.S. Navy Toxicology R & D

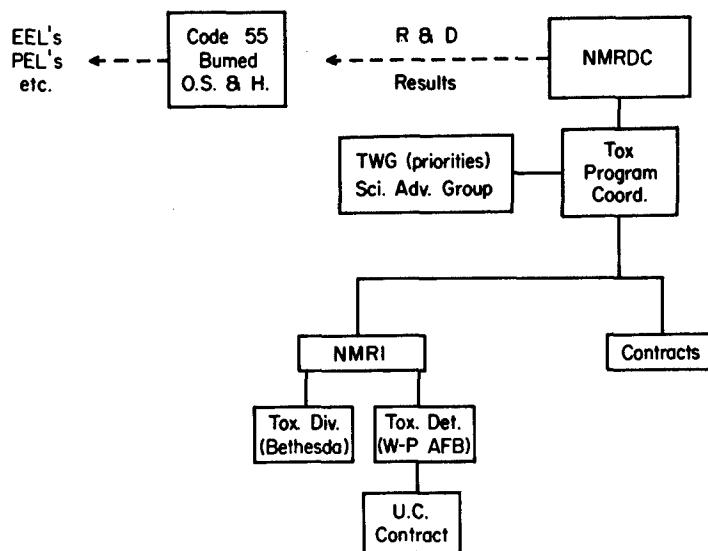


Figure 1.

Moving down from the command level at the NMRDC to the research and development echelons, we note that the total toxicology program is under the direction of a Toxicology Program Coordinator, LCDR L. E. Doptis. He is assisted in this operation by two groups of advisors, a Toxicology Working Group (TWG) and a Science Advisory Group. The TWG is a totally in-house Navy group composed of a series of five panels related to major uses and classes of Navy chemicals, with representatives from the chemical user community and the toxicology research staff. The prime purposes of the TWG are to: (1) prioritize the sequence of toxicological testing of chemicals; and (2) channel information on exposure limits back to the designers and users of military hardware systems. The Science Advisory Group will function in overview on the quality of the toxicology research program and its output.

The actual performance of the research program, still working downward (in Figure 1), is principally by an in-house effort, complemented by some external contracts. The in-house program is directed from the command level of the Naval Medical Research Institute, with responsibility vested in the Environmental Bio-sciences Department of NMRI. At the work unit level of performance, the research program is executed in the Toxicology Division in Bethesda and the newly established Toxicology

Detachment at Wright-Patterson AFB. The Detachment will function as an on-site monitor of progress under the contract with THRU at Wright-Patterson.

Finally, the Navy looks forward with pleasure to a long period of productive collaboration with the Air Force and its prime contractor, the University of California, Irvine, in inhalation toxicological research. We anticipate that the interaction of toxicologists from the Navy, Air Force and the University of California will be highly beneficial to the missions of all the participants in the programs at Wright-Patterson Air Force Base.

ON THE ETIOLOGY AND METABOLIC EPIDEMIOLOGY
OF THE MAIN HUMAN CANCERS*

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INTRODUCTION

It has been stated in scientific as well as lay circles that the majority of human cancers (70-90%) are due to environmental causes (Higginson and Muir, 1973; Higginson, 1976; Wynder, 1976). By environmental causes, it is generally assumed that one means chemical causes, and more specifically, those due to our modern technology and industrial development. In support of this understanding, scientific papers, as well as reports in the public press, indicate that a number of food additives, pesticides, insecticides, and industrial chemicals introduced commercially in the last 40 years have exhibited carcinogenic properties in animal models (Weisburger, 1976). Also, historically, human cancers have been shown to be related to chemical exposure in an occupational environment, the intake of specific drugs, or exposure to specific chemicals

*This paper will appear In: Origins of Human Cancer, J. D. Watson and H. Hiatt (Editors), Cold Spring Harbor Laboratory, Cold Spring Harbor, New York, 1977 (In press).

This work was supported by National Cancer Institute Grants CA-12376, CA-14298, CA-15400 and CA-17613; National Institute of Occupational Safety and Health Grant OH-00611, and Contracts No. 1-CP-33208 (National Cancer Institute) and No. 1-ES-6-2130 (National Institute of Environmental Health Sciences).

(Saffiotti and Wagoner, 1976). Thus, in the public mind, the association between our chemical environment and cancer causation is easily understood. Regrettably, current evidence suggests that the main human cancers, discussed in detail below, do not stem from intentional or even inadvertent chemical contaminants in our environment. We say regrettably because, if such an association could actually be demonstrated, prevention of the main human cancers through removal of the offending substances would be a rather readily achieved goal.

In order to acquire insight into the causes of cancer, it is necessary to analyze the conditions inherent in the occurrence of each specific type of cancer. On the basis of world-wide statistics illustrating the incidence of diverse cancers, the altered risk of migrants from areas of high to low incidence over several generations and the corresponding analysis of data obtained under controlled conditions in animal models, a picture emerges permitting delineation of the multiple causative factors involved in each of the main cancers (Wynder and Mabuchi, 1972; Fraumeni, 1975). According to current ideas, these causal factors are basically unrelated to food additives, insecticides, pesticides, or synthetic contaminants in drinking water. The exception, of course, is the occurrence of specific cancers due to exposure in an occupational setting or the consumption of drugs known to be carcinogenic. It would appear that, at most, 5% of human cancers are due to such exposures (Weisburger, 1976; Higginson, 1976).

The one cogent argument we can marshal for these views depends on two facts: The introduction of a carcinogen to the general public namely, cigarette smoking, mostly in males in the United States during and after World War I, and in females during and after World War II, has resulted 15-25 years later in an impressive rise in the incidence of lung cancer and, secondarily, in an increase in other specific cancers, such as those of the renal excretory pathway (Wynder and Hecht, 1976; Wynder, Gori and Hoffmann, 1976) (Figures 1 and 2). On the other hand, the introduction of insecticides, pesticides, and food additives in the last 40 years appears to have had no detectable effect on the incidence of the main human cancers in the United States, such as those in the breast, prostate and colon (Seidman, Silverberg and Holleb, 1976). Breast and colon cancer have exhibited a slightly increased incidence, almost paralleling the increased intake of daily dietary fat in the United States (Gortner, 1975). The incidence of pancreatic cancer also appears to have risen in the last few years for reasons that are as yet unclear, although one risk factor is smoking and another

is dietary fat (Wynder, 1975; Seidman, Silverberg and Holleb, 1976). On the other hand, stomach cancer has decreased appreciably, as has primary cancer of the liver.

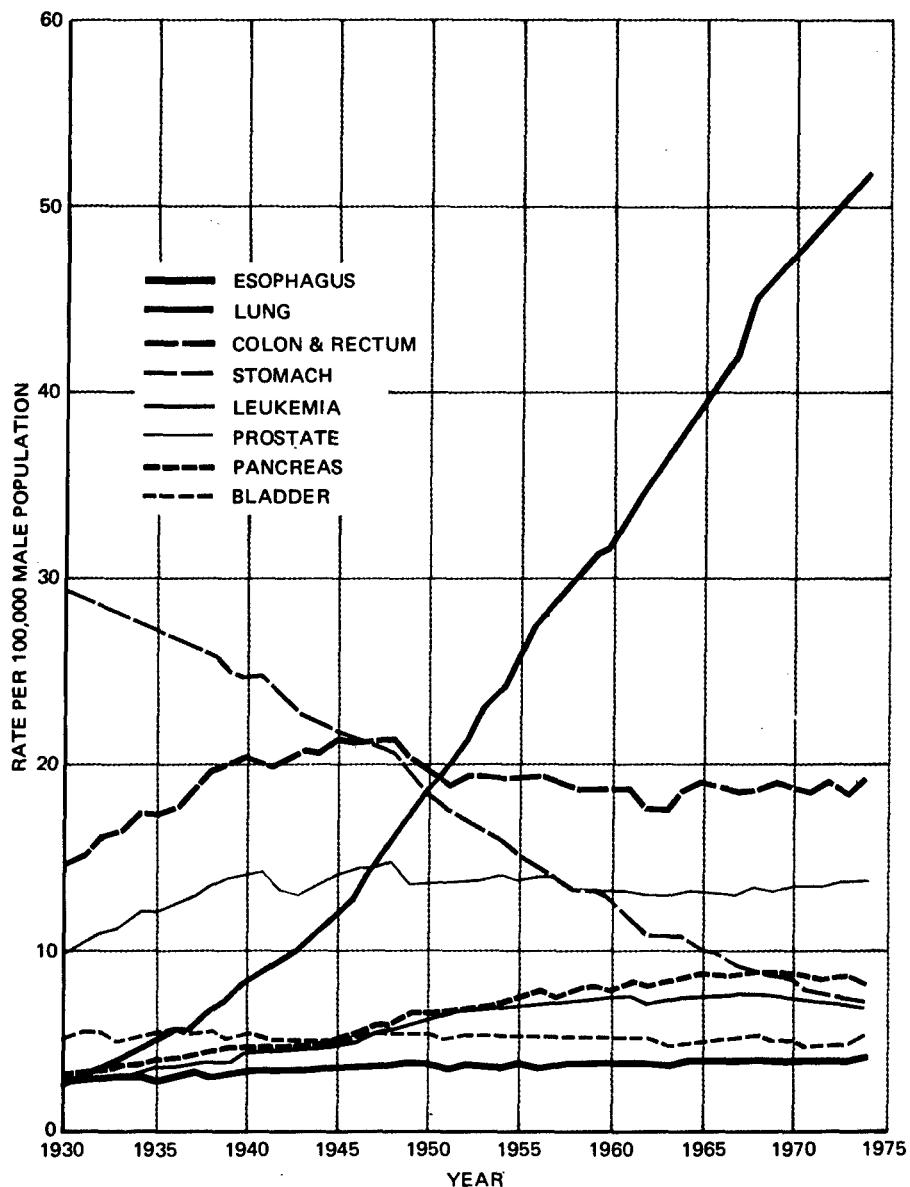


Figure 1. Age-adjusted cancer death rates in males for selected sites (U.S., 1930-1974) (From Seidman, Silverberg and Holleb, 1976)

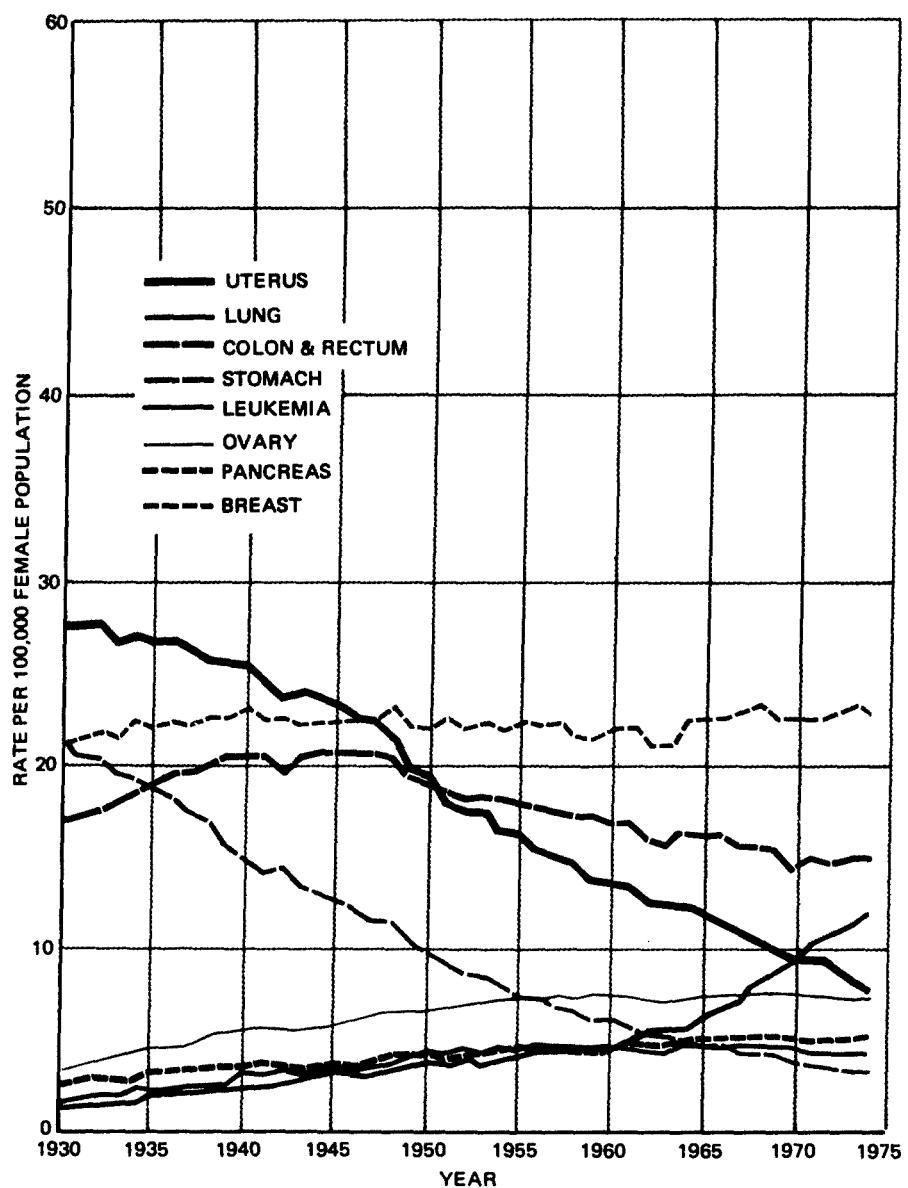


Figure 2. Age-adjusted cancer death rates in females for selected sites (U.S., 1930-1974) (From Seidman, Silverberg and Holleb, 1976).

Below, we are presenting summary statements of the factors contributing to the etiology of the main human cancers in the United States, specifically, cancer of the lung, breast, prostate, stomach and colon.

CANCER OF THE LUNG AND OTHER TOBACCO-RELATED DISEASES

Before 1930, lung cancer was a relatively rare disease. However, since then, its incidence began to increase considerably in the United States for men and since about 1950 also for women (U.S. Surgeon General's Report, 1964, *The Health Consequences of Smoking*; U.S. Government Publ., 1968, 1969, 1971-1974; Hammond, 1976; Wynder and Hecht, 1976; Wynder, Gori, and Hoffman, 1976; Seidman, Silverberg and Holleb, 1976).

The death rate from lung cancer (standardized for age distribution of the U.S. population in 1940) has risen from about 4 per 100,000 in 1930 to 52 per 100,000 in 1975 and the age-adjusted death rate in women climbed from 2 per 100,000 to 11 per 100,000 in the same time (Seidman, Silverberg and Holleb, 1976). It is well established that the primary reason for this dramatic increase in lung cancer incidence and deaths in the U.S. is the smoking of manufactured cigarettes, a practice which men began around the time of World War I and which became fashionable for women just prior to World War II. Data on lung cancer trends in other countries with accurate statistics for lung cancer mortality, such as England and the Scandinavian countries and Japan, also bear out the direct relationships to smoking habits in men and women, besides reflecting variances in availability of cigarettes at certain times (during wars) (Doll and Hill, 1964; Kreyberg, 1969; Segi and Kurihara, 1972).

The strong statistical associations of cigarette smoking and lung cancer incidences, chiefly developed by Wynder, Doll and Hammond have prompted laboratory investigations on the carcinogenic potential of tobacco smoke since 1953 (Synder, Graham and Croninger, 1953). The biological evaluation of tobacco smoke and its condensate, together with the chemical identification of the major components involved in tobacco carcinogenesis have not only contributed much information but have led also to product modifications aimed at less harmful cigarettes (Gori, 1976; Hammond, 1976; Hammond et al., 1976). In the United States, major laboratory work was initiated by Wynder in 1953, and contributions have been made and continue to emanate from work by Wynder and Hoffmann, Bock, Van Duuren, Homburger and Gori. These and worldwide contributions are reflected in a book on Experimental Tobacco Carcinogenesis (Wynder and Hoffmann, 1967) and, more recently, in the Proceedings of the 3rd World Conference on Smoking and Health (Wynder, Hoffmann and Gori, 1976), and also in the monograph "Lung Cancer" (Wynder and Hecht, 1976).

Tobacco smoke is an aerosol consisting of a gaseous and a particulate phase with a distribution equilibrium of certain smoke constituents in both phases. As a respiratory carcinogen, with a median particle size of 0.4μ , the aerosol exerts multiple effects of gaseous as well as particulate components. Smoke constituents play a role as ciliotoxins, as initiators of the carcinogenic process, as cocarcinogens and tumor promoters (Hoffmann et al., 1976). In animal models, tobacco carcinogenesis has been studied in a variety of species and sites. Tumors have been induced with smoke particulates on mouse skin, rabbit ear, mouse cervix, in newborn mice and also in the connective tissue, trachea and lungs of rats and in the trachea of dogs. In the upper respiratory tract of hamsters, mice and rats, tumors have been elicited as a response to spraying of the smoke condensates as aerosols (Hoffmann et al., 1976). Tobacco smoke particulates and fractions thereof have also been found to have mutagenic effects in several strains of *Salmonella* as was demonstrated in Ames' group (Kier, Yamasaki and Ames, 1974) and by Sugimura et al., 1976.

The gaseous phase of cigarette smoke condensate has not induced tumors in animal models, but it has been shown to contain trace amounts of carcinogens, such as the volatile nitrosamines and also tumor promoters, such as formaldehyde and major ciliotoxic agents, such as hydrogen cyanide, formaldehyde, acrolein and acetaldehyde (Table 1).

While animal models and passive inhalation techniques for a study of respiratory carcinogens have now been developed to a greater degree, they do not appear to circumvent one chief obstacle in tobacco smoke carcinogenesis, the natural defense of obligatory nosebreathers, against particles in inhaled aerosols. Much of the particulate is filtered from the smoke and fails to reach the lungs. Nonetheless, neoplastic lesions in the larynx have been obtained by chronic exposure of hamsters to cigarette smoke. More direct introduction of tobacco smoke into the trachea of dogs by a tracheotomy has led to early lesions in the lung in as short a time as three years (Auerbach et al., 1967, 1970).

TABLE 1. TUMORIGENIC AGENTS IN THE GAS PHASE OF TOBACCO SMOKE^a

<u>Toxic Agents</u>	<u>Concentration in smoke of one cigarette</u>	
I. Carcinogens ^b		
$\text{H}_3\text{C}-\text{N-NO}$	5 -	180 ng
$\text{R}_1-\text{N-NO}$ (6 compounds)	2 -	200 ng
$\text{H}_2\text{N-NH}_2$	24 -	43 ng
$\text{H}_2\text{C=CHCl}$	10 -	40 ng
II. Tumor Promoters		
HCHO	10 -	90 µg
III. Cilia Toxic Agents		
HCN	100 -	700 µg
HCHO	20 -	90 µg
$\text{H}_2\text{C=CH-CHO}$	45 -	140 µg
$\text{H}_3\text{C-CHO}$	18 -	1440 µg

^aList is based only on publications with unambiguous identifications of toxic agents (From Wynder and Hoffman, 1976).

^bTobacco smoke is suspected of also containing H_3As , $\text{Ni}(\text{CO})_4$, and possibly volatile chlorinated olefins and nitro-olefins.

The complexity of the particulate phase of tobacco smoke was unraveled by fractionation techniques which have led to the identification of the major tumorigenic components and their relative roles in bioassays. These compounds are listed in Tables 2-4.

TABLE 2. TUMOR INITIATING AGENTS IN THE PARTICULATE PHASE OF TOBACCO SMOKE^a

<u>Compound</u>	<u>Relative Activity as Complete Carcinogen^b</u>	<u>ng/cig^c</u>
Benzo(a)pyrene	+++	10 - 50
5-Methylchrysene	+++	0.6
Dibenz(a,h)anthracene	++	40
Benzo(b)fluoranthene	++	30
Benzo(j)fluoranthene	++	60
Dibenzo(a,h)pyrene	++	pr
Dibenzo(a,i)pyrene	++	pr
Dibenzo(a,j)acridine	++	3 - 10
Ieno(1,2,3-cd)pyrene	+	4
Benzo(c)phenanthrene	+	pr
Benzo(a)anthracene	+	40 - 70
Chrysene	+	40 - 60
Benzo(e)pyrene	+?	5 - 40
2-, 3-Methylchrysene	+	7
1-, 6-Methylchrysene	-	10
2-Methylfluoranthene	+	34
3-Methylfluoranthene	?	40
Dibenz(a,c)anthracene	(+)	pr
Dibenzo(a,h)acridine	(+)	0.1
Dibenzo(c,g)carbazole	(+)	0.7

^aIncomplete list; all mentioned compounds are active as tumor initiators on mouse skin (From Hoffmann et al., 1976).

^bRelative carcinogenic activity on mouse skin as measured in our own laboratory on Swiss albino (ha/ICR/Mil) mice.

? - Carcinogenicity unknown.

(+) - Not tested in our own laboratory.

^cpr - Present, but no quantitative data given.

TABLE 3. COCARCINOGENIC AGENTS IN THE PARTICULATE MATTER OF TOBACCO SMOKE^a

<u>Compound^b</u>	<u>Cocarcinogenic Activity^c</u>	<u>ng/cig^d</u>	
I. Neutral Fraction			
Pyrene (-)	+	50	-
Methylpyrenes (?)	?	50	-
Fluoranthene (-)	+	100	-
Methylfluoranthene (+;?)	?		180
Benzo(ghi)perylene (-)	+		60
Benzo(e)pyrene (+)	+		30
Other PAH (+)	?		?
Naphthalenes (-)	+	360	-
1-Methylindoles (-)	+		830
9-Methylcarbazoles (-)	+		140
4,4'-Dichlorostilbene (-)	+		1,500
Other neutral compounds (?)	?		?
II. Acidic Fraction			
Catechol (-)	+	200,000	- 500,000
4-Alkylcatechols (?)	?	>	10,000
Other phenols (?)	?		?
Other acidic agents (?)	?		?

^aIncomplete List (From Hoffmann et al., 1976).

^bIn parenthesis, carcinogenic activity on mouse skin.

(?) - Unknown.

^c+ - Active.

? - Unknown.

^dValue from 1968 USA cigarette; today values will be lower, because DDT and DDD decreased in US tobaccos.

TABLE 4. ORGAN SPECIFIC CARCINOGENS IN TOBACCO SMOKE PARTICULATES^a

	<u>Carcinogen</u>	<u>Conc/cig</u>	<u>Carcino- genicity^b</u>
I.	<u>Esophagus</u>		
	N'-Nitrosonornicotine	137 ng	+
	Nitrosopiperidine	0 - 9 ng	
	Unknown unsymmetrical nitrosamines	?	+
II.	<u>Lung</u>		
	Polonium-210	0.03-1.3 pCi	+
	Nickel compounds	0 - 600 ng	+
	Cadmium	9 - 70 ng	
	Unknowns	?	?
III.	<u>Pancreas</u>		
	Nitrosamines	?	+
	Unknowns	?	?
IV.	<u>Kidney and Bladder</u>		
	β -Naphthylamine	22 ng	+
	α -Aminofluorene	+	+
	α -Aminostilbene	+	+
	α -Toluidine	+	+
	Unknown aromatic amines	?	?
	α -Nitrotoluene	21 ng	?
	Unknown nitro compounds	?	?
	Di-n-butylnitrosamine	0 - 3 ng	+
	Other nitrosamines	?	+

^aList is incomplete; data are based on experimental data only (From Hoffmann et al., 1976).

^bAnimal data on carcinogenicity.

It was shown that the major carcinogenic activity on mouse skin was due to the neutral components (mainly polynuclear aromatic hydrocarbons), although cocarcinogens in this fraction as well as tumor promoters in the weakly acidic portion and in the basic portion of the smoke condensate further contribute to the total carcinogenic effects. The structural elucidation of tobacco smoke constituents with biological activities has more recently led to the finding of tobacco-specific nitrosamines which are derived from tobacco alkaloids. Testing of synthetic alkaloid-nitrosamines has shown carcinogenicity in rats and in the upper respiratory tracts of the Syrian golden hamster for N'-nitrosonornicotine (Hilfrich, Hecht and Hoffmann, 1977). Since compounds of this type are found in chewing tobacco in concentrations up to 90 ppm (Hoffmann et al., 1974), a further evaluation of their role in human carcinogenesis appears warranted. The possible *in vivo* nitrosation of tobacco constituents which would lead to the formation of organ specific carcinogenic nitrosamines is an area of investigation that requires further attention.

The presence in tobacco smoke particulates of aromatic amines and other bladder carcinogens and the statistical association of bladder and kidney cancer mortality with cigarette smoking (Hammond, 1975) demands a structured and systematic approach towards the elucidation and quantitation of biologically active compounds.

While the association of tobacco smoking and lung cancer is a major one, and is therefore the one most extensively studied, the risk factors for cancer of the kidney and urinary bladder, cancer of the pancreas, cancer of the upper alimentary tracts, as well as emphysema and coronary heart disease in smokers must be more thoroughly identified (Hammond, 1975). The study of such risk factors and of the pathogenesis of the associated diseases is a prerequisite to preventive intervention. It has been estimated that 40% of male premature deaths are due to diseases associated with the habit of smoking cigarettes (Hammond, 1975). In view of the less than totally successful attempts to eliminate smoking, the modification of tobacco products towards less harmful cigarettes was a commendable step. Cigarettes characterized by low-tar and low-nicotine contents are now available and are becoming increasingly popular, due to efforts in educating the public through statements such as the Surgeon General's Report and educational campaigns by the American Cancer Society and other national organizations (Wynder and Stellman, 1977).

Human evidence appears to indicate a trend towards reduced cancer incidence and mortality rates (Wynder, 1972; Wynder and Hecht, 1976; Wynder, Mushinski and Stellman, 1976) in line with the reduced exposure to cigarette smoke constituents since educational campaigns and product modifications have begun two decades ago. Laboratory data reflect a reduction of harmful constituents in cigarette smoke and smoke particulates exhibit lesser degrees of tumorigenicity in bioassays than the corresponding products did one or two decades ago (Gori, 1976).

However, a cigarette engineered with all technical refinements known today is not as effective in diminishing the risk of disease as is total smoke cessation. Thus, efforts of smoke withdrawal clinics, such as those associated with the American Health Foundation and the American Cancer Society, must be continued. More importantly, education of the young people must be stressed so as to clearly present the risk factors and to motivate them never to smoke at all.

Current data show that a formerly heavy smoker who stops his habit will decrease his risk of cancer progressively and, after 15 years, his risk factors are considered to be nearly those of someone who never smoked (Hammond, 1975; Wynder and Hecht, 1976).

The underlying reason for this risk reduction is thought to relate to the fact that tobacco carcinogenesis is mainly due to cocarcinogenic and tumor promoting factors which exert their effects in the long term smoker only because of their continuous presence.

In this context, it is important to stress tobacco smoking as a habit potentiating occupational cancer risks (Hoffmann and Wynder, 1976) as well as other lifestyle factors.

In association with heavy drinking of alcoholic beverages, smoking also leads to cancer in the oral cavity and the esophagus (Schottenfeld, Gantt and Wynder, 1974). In the United States, this appears to be more prevalent in lower socio-economic groups. In other areas of Western Europe such as in France, the disease is also regionalized and appears to be highest in smokers who drink certain concentrated alcoholic beverages, such as calvados (Day, 1975). Preliminary experiments by Dr. G. D. McCoy in our Institute suggest that the malnutrition resulting from consumption of alcoholic beverages may alter the target organ for respiratory carcinogens through one or more mechanisms such as:

1. induction of carcinogen-activating enzymes; 2. nutritional deficiencies, particularly of vitamins, and other factors leading to shifts in cell energetics and oxidative mechanisms; or 3. a promoting or cocarcinogenic effect of metabolites of alcohol.

Thus, the study of tobacco carcinogenesis has, as yet, to resolve many underlying questions with regard to the total adverse health effects associated with tobacco smoking.

CANCERS ASSOCIATED WITH DIET

Several lines of evidence suggest that cancers of the digestive tract, and in particular, cancer of the stomach, colon and pancreas, are associated with dietary components. Furthermore, cancers of the endocrine-related organs, such as breast, prostate, ovary and endometrium, are also linked to dietary factors. With the exception of lung cancer, discussed above, these are the human cancers with the highest incidence in the United States. A recent symposium dealt in detail with the relation between nutrition and cancer (Wynder, Peters and Vivona, 1975).

Knowledge in these areas stems from observations of cancer incidence in various parts of the world. For example, it is noted that cancer of the stomach is prevalent in Japan, the Western part of South America, parts of Central America, and Northern and Eastern Europe, while it has a low incidence in the United States. In addition, the lower socio-economic groups have a higher incidence of stomach cancer than more affluent groups. On the other hand, the pattern of cancer of the colon, breast, prostate, pancreas, ovary and endometrium exhibits almost the opposite picture, being high in the United States, Western Europe, Australia, New Zealand and Argentina. This cancer distribution has been shown to have origins in the environment rather than genetic differences between populations through a study of the changes in incidence found in migrant groups in the first or second generation. For example, Japanese migrants have a decreasing risk for gastric cancer and an increasing risk for colon and breast cancer. In light of the high degree of industrialization in Japan, it is probably true that industrial activity per se is not responsible for the etiology of the main human cancers.

People in Northern or Eastern Europe or Japan, with a high risk for gastric cancer in successive generations in the United States, do not present this risk, but instead show cancer in the colon, breast or prostate. It is sometimes stated, with fatalistic resignation, that man is destined to have cancer of one type or the other. It will be shown that the etiological factors for gastric cancer are of a totally different nature than those for colon or breast cancer and, thus, can be controlled independently of those factors leading to the latter types of cancer. With developing knowledge in this field, it can be hoped and expected that the risk for gastric cancer can be reduced without necessarily incurring a simultaneous risk for the other cancers.

The key factors currently considered to be responsible for the cancers discussed below are dietary in nature. They are specific micronutrients and components (gastric cancer, perhaps also liver and esophageal cancer in certain populations) or macronutrients (colon, breast, prostate, ovary, endometrium and perhaps pancreas cancer). Further research efforts are required to pinpoint the underlying mechanism and secure data on interactions between macro- and micronutrients which might modulate and even control the overall effect.

GASTRIC CANCER

In many countries of the world, gastric cancer is or was once a major neoplastic disease of humans. In the United States, there has been a pronounced decrease in the incidence of this disease in both men and women during the last 40 years.

Epidemiological data show that gastric cancer incidence is high in Japan and Eastern Europe, compared to the United States (Wynder et al., 1963; Haenszel and Correa, 1975). The theory that gastric cancer would be attributable to dietary or environmental factors is supported by findings from the study of Japanese migrants to Hawaii. While the first generation of Japanese residing in Hawaii showed almost the same gastric cancer incidence as native Japanese, the second generation showed a pronounced trend to the low incidence rates of the Hawaiians (Haenszel, 1975).

Numerous hypotheses have been proposed, mostly concerning diet, to account for the etiology of this important cancer. Thus, the consumption of foods high in oxidized fats or the intake of smoked fish have been incriminated in the past (Higginson, 1967; Haenszel and Correa, 1975), but experimental support for these suggestions is unconvincing.

Until recently there were no reliable animal models for the study of this cancer (Bralow and Weisburger, 1976). Sugimura, however, demonstrated that chemicals of the group of alkylnitrosoureas and, particularly, alkyl-N'-nitro or N'-acyl N-nitrosoureas induced gastric cancer in many species (Sugimura and Kawachi, 1973). Sander and Schweinsberg (1972) observed that such chemicals could be formed from suitable substrates and nitrites at the pH of the stomach. Mirvish (1975) reported on the kinetics of this reaction which can undergo positive catalysis by agents such as thiocyanate (Fan and Tannenbaum, 1973; Boyland and Walker, 1974). On the other hand, Vitamin C powerfully inhibited the nitrosation reaction (Mirvish et al., 1972; Raineri and Weisburger, 1975).

We have asked the question whether the endogenous formation of such chemicals may be responsible for human gastric cancer (Weisburger and Raineri, 1975). In the United States, sizeable amounts of nitrate and salt were used as food preservatives, especially for fish and meat, prior to the advent of refrigeration. This is no longer necessary since such foods are now preserved through routine storage at low temperatures. Currently, also, federal regulations limit the amounts of nitrate and nitrite used for this purpose. In fact, there has been a tendency toward eliminating nitrates altogether. Thus, in the United States, there has been a sizeable decrease in dietary nitrate intake. On the other hand, in some regions of the world such as Central and South America the soil, and hence the agricultural products grown on such soil, contains large amounts of nitrate. Some well waters, likewise, are high in nitrate. Such nitrate is reduced to nitrite during storage. Thus, in various parts of the world, (e.g., United States) there are now or have been appreciable amounts of nitrite in the dietary environment. In their migrant studies, Haenszel et al. (1975) and Correa et al. (1975) noted that the risk for gastric cancer is lower in those people eating more fruits and lettuce, both sources of Vitamin C. This vitamin has been shown experimentally to inhibit nitrosation reactions, and hence, the production of potential carcinogens of the type described.

In the search for the etiologic factors responsible for gastric cancer, we have examined extracts of nitrite-treated foods for mutagenic activity in different test-strains of Salmonella typhimurium (Ames, McCann and Yamasaki, 1973). Foods tested were those consumed predominantly in regions with high stomach cancer incidence. Extracts of fish, borscht and beans, which are staples of the diet in Japan, Eastern Europe, or Latin America, showed mutagenic activity upon treatment with nitrite. In contrast, typical American foods like hot dogs or beef, failed to develop mutagenic activity with nitrite, perhaps because nitrite reacts preferentially with myoglobin (Marquardt, Rufino and Weisburger, 1977a,b) (Table 5).

TABLE 5. MUTAGENIC EFFECTS OF EXTRACT OF NITRITE-TREATED FOOD IN SALMONELLA TYPHIMURIUM TA 1535 PLATE INCORPORATION ASSAY AS A FUNCTION OF DOSE PER PLATE

<u>Extract</u>	<u>Doses/plate (μl)</u>	<u>No. of histidine positive revertant colonies</u>
Fish ^a	1	70 + 12.7
	5	184 + 19.8
	10	252 + 29.0
	20	212 + 5.7
	40	Killing
Beans ^b	1	26 + 1.4
	10	59 + 5.7
	25	73 + 0
Borscht ^b	1	26 + 7.1
	10	29 + 3.5
	25	65 + 0

^aSpontaneous revertants 8 + 2.8.

^bSpontaneous revertants 6 + 1.4.

(From Marquardt, Rufino and Weisburger, 1977).

The formation of the mutagen was maximal at pH 3. Storage under highly acidic conditions, as well as under alkaline conditions, destroyed the mutagen(s). In a dose-response study, incubation with 5000 ppm of sodium nitrite yielded the highest amount of mutagenic activity, but activity was also observed with 1000 ppm of nitrite. The mutagenic material did not require metabolic activation. Moreover and importantly, ascorbic acid prevented the formation of the mutagen(s) in nitrite-treated foods. These data suggest that the mutagen(s) may be of the alkynitrosamide-type.

In 1975, Endo et al. showed that nitrosation of methylguanidine, under simulated gastric conditions, generated a mutagenic principle identified as nitrosocyanamide (Endo et al., 1974, 1975). However, it was found recently that fish and other foods probably do not contain significant amount of methylguanidine (Fujinaka et al., 1976). Also, nitrosocyanamide, while highly mutagenic, induced mainly forestomach tumors in rats and thus fails to exhibit the specificity of inducing cancer in the glandular stomach, as seen in man, which chemicals like N-methyl-N'-nitro-N-nitrosoguanidine do produce reliably.

Therefore, identification of mutagenic agents in nitrosated foods requires further study. Also, the direct relevance of our findings for human gastric carcinogenesis remains to be demonstrated. Animal experiments investigating the *in vivo* carcinogenic properties of the mutagenic principle found in the extracts of fish, borscht and beans are underway.

If the concept that gastric cancer stems from an *in situ* nitrosation of endogenous substrates is borne out experimentally, it would appear that one relatively minor change in the human diet in high risk groups would prevent this important cancer. The required alteration would make available fruits, vegetables and salads as sources of Vitamin C on a continuous, rather than intermittent seasonal basis, as is presently the custom in many countries. It is necessary to emphasize the need for a continuous dietary intake of foods with Vitamin C to prevent even an intermittent exposure to carcinogens, inasmuch as in animal models, gastric cancer can be induced by relatively infrequent application of alkynitrosamides. Also, epidemiological data indicate that first generation migrants from high risk countries like Japan, Poland or Scandinavia maintain the risk for gastric cancer in their adopted country, suggesting that once initiated, the reaction proceeds. Hence, there is a need to avoid formation of gastric carcinogens early in life and continue this practice by minimizing intake of actual or potential nitrite, and optimizing the intake of foods containing ascorbate. On the basis of our evidence, that meats in the presence of nitrite do not generate mutagens but that nitrite and fish or beans do, we can conclude that addition of small amounts of nitrite to meats as a preservative measure, as now practiced, is probably not hazardous. On the other hand, populations eating mainly fish need to minimize environmental nitrite and ensure the regular presence of ascorbate in their daily diet.

RECTAL CANCER

In the past, rectal cancer was considered often together with colon cancer, as "colo-rectal" or "large bowel" cancer. There begins to be a realization that these may be two separate diseases. A better distinction is afforded by the more precise anatomic localization, with rectal cancer involving tissue within 8 cm of the anus. Thus, defined, colon cancer has exhibited a small increase in rate over the last 30-40 years, paralleling an increased intake of dietary fat (Gortner, 1975), but rectal cancer has decreased. Further, the male/female ratio for colon cancer is about 1/1, for rectal cancer 1.4/1. Also, the incidence in a high risk versus a low risk country, like the United States - Japan, is about 514 times higher for colon cancer, but only 1.5 times higher for rectal cancer. Etiologic factors for rectal cancer remain to be defined.

COLON CANCER

On the basis of variations in incidence for different regions of the world and in view of the altered risk of migrant populations, it has been accepted that diet is a major etiologic factor in colon cancer (Correa, 1975). Further epidemiologic evaluation has implicated a high intake of dietary fat (Wynder and Shigematsu, 1967; Wynder et al., 1969; Wynder and Reddy, 1975), protein (Armstrong and Doll, 1975), beef (Haenszel et al., 1973), and possibly dietary fiber deficiency (Burkitt, 1975) as strongly associated with large bowel carcinogenesis. A major portion of the dietary fat in high risk areas is derived from meat and, in particular, beef. Also, diets high in fat are often low in fiber. On the basis of epidemiologic and laboratory evidence, we have emphasized dietary fat, but new efforts are underway to round out knowledge on other dietary components suspected of playing a role in large bowel carcinogenesis.

The typical American diet contains 40-45% of calories as fat, direct and hidden (in meats). In Japan, fat, more of it unsaturated, accounts for only 15-20% of daily calories, which are, in turn, about 10% lower than in the United States.

Most of the research has been concerned with the possible etiological role of fats, especially since they affect cholesterol and bile salt metabolism and excretion, as well as colonic bacterial flora. To account for the relationship between dietary fat and colon cancer, it has been postulated that (a) the amount of dietary fat determines both the concentration of acid and neutral sterol substrates in the large bowel and also the composition of the microflora acting on such substrates; and (b) the gut microflora metabolize acid and neutral sterols to carcinogens or cocarcinogens active in the large bowel (Hill et al., 1971; Wynder and Reddy, 1973; Hill, 1975). Such microflora-mediated reactions may also yield products which may be metabolized further to a carcinogen by the intestinal mucosa itself.

Burkitt (1975) and Painter and Burkitt (1975) have emphasized dietary fiber and consequent volume of intestinal content and size of stools in relation to several diseases of the intestine and other conditions. While diverticulitis, for example, may stem in part from lack of dietary fibers, current views are that colon cancer is unrelated to fiber. Wynder and Shigematsu (1967) failed to find an association between constipation and colon cancer, and experimental models so far have not provided positive data (Ward, Yamamoto and Weisburger, 1973), whereas as discussed below, fat levels do influence colon carcinogenesis.

Hill et al. (1971) observed a correlation between the death rate due to colon cancer in various populations and the fecal excretion of metabolites of cholesterol and bile acids as well as their degradation products by bacterial flora. Reddy and Wynder (1973) similarly found that populations on a mixed Western diet, among whom the rate of large bowel cancer is high, degraded and excreted acid and neutral sterol metabolites to a greater degree than a similar population with a comparatively low rate of colon cancer (Figure 3). The fecal bacteria of groups consuming a mixed Western diet also had higher β -glucuronidase activity. These differences are associated with dietary composition, mainly with a higher content of animal fat and protein in the high risk group. Controlled studies, comparing a high meat, high fat diet with a meatless, low fat diet showed that the former resulted in an elevated level of fecal bile acid and cholesterol metabolites, increased bacterial β -glucuronidase activity, and more total microflora (Reddy, Weisburger and Wynder, 1974; Reddy, Weisburger and Wynder, 1975).

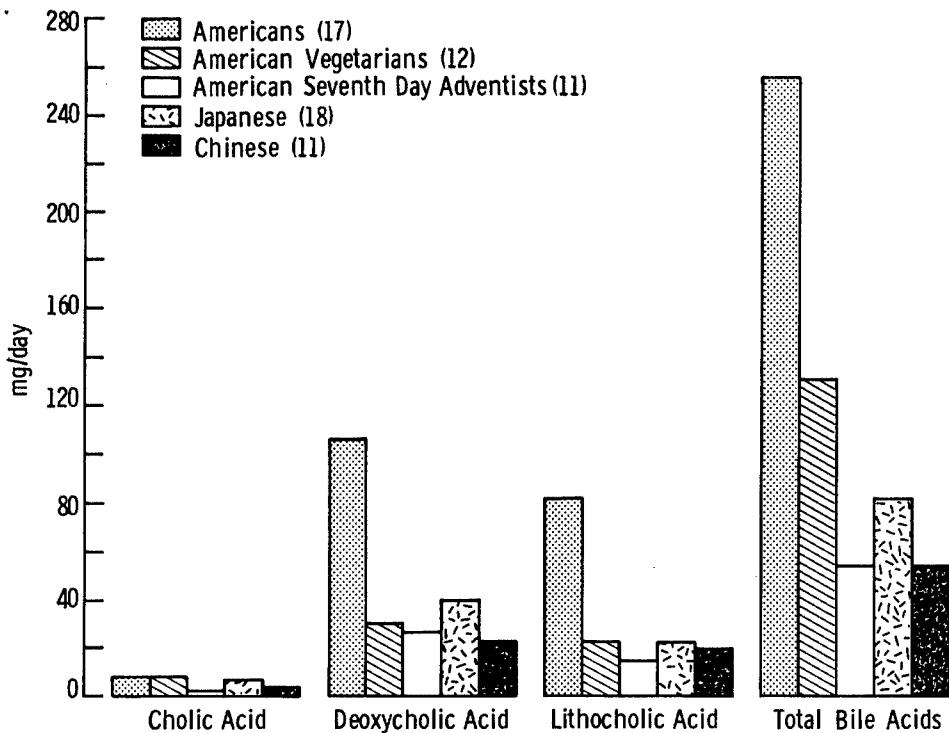


Figure 3. Excretion of fecal bile acids in populations with differing risk for colon cancer. (From Reddy and Wynder, 1973).

Comparisons of fecal constituents in terms of bacteria, cholesterol, bile acid metabolites and bacterial enzymes were performed on patients with colon cancer. In a case control study, Hill et al. (1975) found that patients with colon cancer had increased levels of fecal bile acids and nuclear dehydrogenating Clostridia, compared to controls. Reddy and Wunder (1977) and Mastromarino, Reddy and Wynder (1976) have also shown that the concentration of bile acid and cholesterol metabolites in colon cancer patients was higher than in controls, as was the fecal bacterial 7 α -dehydroxylation of primary bile acids (Figure 4).

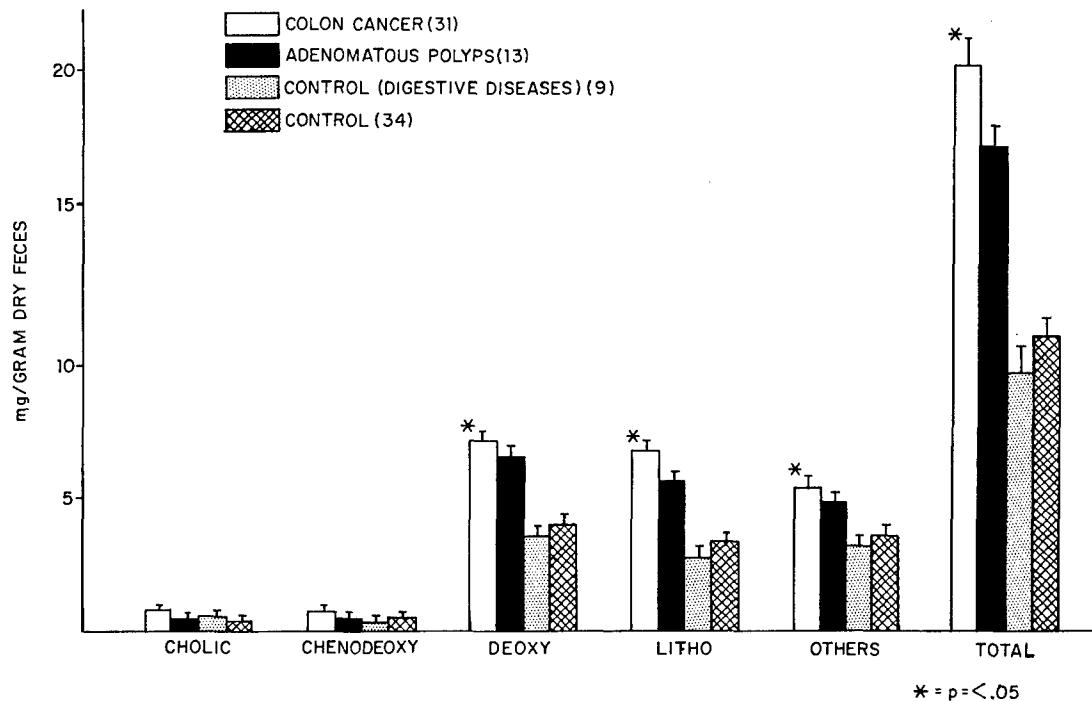


Figure 4. Excretion of bile acids in patients with colon cancer, adenomatous polyps, or other digestive diseases and controls. (From Reddy and Wynder, 1977).

It is generally agreed that patients with familial polyposis, ulcerative colitis, and adenomatous polyps have an increased risk of developing carcinoma of the colon.

Based on studies thus far conducted in our laboratories (Reddy et al., 1976a; Reddy and Wynder, 1977), those patients at high risk for colon cancer fall into three distinct groups in regard to their fecal bile acid and cholesterol metabolite profiles. Patients with adenomatous polyps excreted high levels of both cholesterol metabolites and bile acids compared to controls; patients with familial polyposis excreted similar levels of total neutral sterol and bile acids, but significantly more unchanged cholesterol compared to controls; and ulcerative colitis patients excreted high levels of neutral sterols but similar levels of bile acids compared to controls. It is evident from these studies that the common denominator in all cases is cholesterol metabolites.

Animal Models for Colon Cancer

Research on the mechanisms of cancer causation in the large bowel has been assisted by the discovery, during the last 20 years, of several animal models which mirror relatively faithfully the type of lesions seen in man. These models involve the induction of large bowel cancer by (1) chemicals of the types 3-methyl-4-aminobiphenyl or 3-methyl-2-naphthylamine, tested so far only in rats and hamsters (less effective); (2) derivatives and precursors of cycasin and methyl-azoxymethanol, such as azoxymethane and 1,2-dimethylhydrazine, which work well in rats, mice of select strains and hamsters; (3) intrarectal administration of direct-acting carcinogens such as methylazoxymethanol acetate, or alkynitrosoureas, such as N-methyl-nitrosourea or N-methyl-N'-nitro-N-nitrosoguanidine, which induce cancer of the descending large bowel in every species tested thus far; and (4) the oral administration of large doses of 3-methylcholanthrene which induces large bowel cancer in select strains of hamsters (Bralow and Weisburger, 1976).

The modes of action of these various carcinogens, which may also bear on human cancer causation, has received only limited study and further efforts in this area are being pursued in a number of laboratories, including our own (Zedeck et al., 1970; Spjut and Noall, 1971; Hawks and Magee, 1974; Laqueur and Spatz, 1975; Fiala et al., 1976, 1977). Specifically, we are interested in the question why ortho-methylarylaminies appear to exhibit organotropism for the colon in rodents, whether such chemicals are present in our environment, and thus be responsible, in part, for large bowel cancer in man. We are further studying the metabolism and mode of action of 1,2-dimethylhydrazine, and the derived azoxymethane and methyl-azoxymethanol by the use of radioisotope and specific separation and analytical techniques. Only small amounts of a given dose are secreted in the bile, and the metabolites secreted may bear on the development of duodenal tumors, but probably not on tumors in the large intestine. The latter seems to relate to specific metabolites brought to the colon by the blood. There is preliminary information that the colon mucosa does not metabolize 1,2-dimethylhydrazine or azoxymethane well, if at all, whereas the liver does (Fiala, 1977; Fiala et al., 1977). The problem, then would be why methyl-azoxymethanol, produced in the liver, exhibits a specific action on the colon.

Modifying Effect of Dietary Fat in Colon Carcinogenesis

The possible role of dietary fat in the induction of human cancer of the large bowel has received some support from experimental studies. Rats fed either 20% lard or 20% corn oil were more susceptible to colon tumor induction by 1,2-dimethylhydrazine and also excreted higher fecal bile acid and cholesterol metabolites compared to those fed 5% lard or 5% corn oil (Reddy et al., 1976b) (Table 6). Recent data by Reddy, Narisawa and Weisburger (1976) indicate that a high meat, high fat diet or a high soy protein, high fat diet lead to more colonic tumors in rats given 1,2-dimethylhydrazine than control diets. On the other hand, fiber in the form of alphacel failed to affect colon carcinogenesis (Ward, Yamamoto and Weisburger, 1973), but we have studies underway with other forms of fiber. Selenium has inhibited colon cancer induction by, as yet, unknown mechanisms (Jacobs, Jansson and Griffin, 1977).

TABLE 6. TUMOR INCIDENCE IN RATS TREATED WITH DMH AND FED TWO LEVELS OF DIETARY CORN OIL OR LARD^a

Diets	Animals with Tumors (%)				multiple colonic tumors (%)	Total colon tumors (per rat)
	Ear Canal	Kidney	Small In- testine	Colon		
Corn oil, 5%	32	4	27	36	14	0.77
Corn oil, 20%	59	14	36	64	32	1.55
Lard, 5%	13	0	4	17	4	0.22
Lard, 20%	67	0	50	67	29	1.50
Purina Lab Chow	15	0	20	25	0	0.25

^aNumber of animals per group ranged from 20 to 24. Animals received weekly s.c. injections of 10 mg/kg body weight for 20 weeks and were autopsied 10 weeks after the last injection. (From Reddy et al., 1976b).

Role of Bile Metabolites in Colon Carcinogenesis in Animal Models

There is evidence that bile acids can promote colon tumor development. Narisawa et al. (1974) have reported that both taurodeoxycholic acid and lithocholic acid are promoters in conventional rats, while Reddy et al. (1976c) have shown that in germ-free and conventional rats, the secondary bile acids, deoxycholate and lithocholic acid, acted as colon tumor promoters. The effect of the primary bile acids, cholic acids and chenodeoxycholic acid as tumor promoters was more pronounced in conventional rats compared to germ-free rats. Thus, in this animal model, these secondary bile acids, present in high concentrations in human stools, serve as promoters. Likewise, Nigro, Bhadrachari and Chomchai (1973) and Chomchai, Bhadrachari and Nigro (1974) observed that an increase of bile salts in the colon of rats, induced by either feeding cholestyramine or by surgically diverting bile to the middle of the small intestine, enhanced colon tumor formation.

Further research is required to elucidate the mechanism by which a high fat diet might translate into a high risk for colon cancer. A search for the carcinogens involved, such as in the laboratories of Bruce and Heddle, or Mower et al. (1977), and in our laboratories may provide a basis for blocking the carcinogenic process at the onset by eliminating or, at least, decreasing the level of the agents involved. The recent findings by Sugimura et al. (1977) of certain complex heterocyclic aromatic amines after pyrolysis of proteins and amino acids provide interesting and suggestive leads. With increasingly detailed knowledge of causative factors and the mechanisms involved, it is hoped that this cancer now with such a high incidence can be prevented through multidisciplinary research approaches.

BREAST CANCER

Epidemiological studies show that premenopausal American and Japanese women have comparable rates of breast cancer as a function of age, although Japanese women exhibit a somewhat lower incidence compared to American women (Figure 5). Furthermore, the incidence in Japanese women reaches a plateau around 45 years of age and then decreases (Berg, 1975). In contrast, the incidence in Americans increases sharply during and following menopause, the curve showing a characteristic "hook". A distinct change in the incidence rate of breast cancer after 48 years of age has also been reported in Scandinavian populations (Hakama, 1969). This biphasic incidence of breast cancer in pre- and post-menopausal women possibly reflects two independent disease factors (DeWaard, 1975).

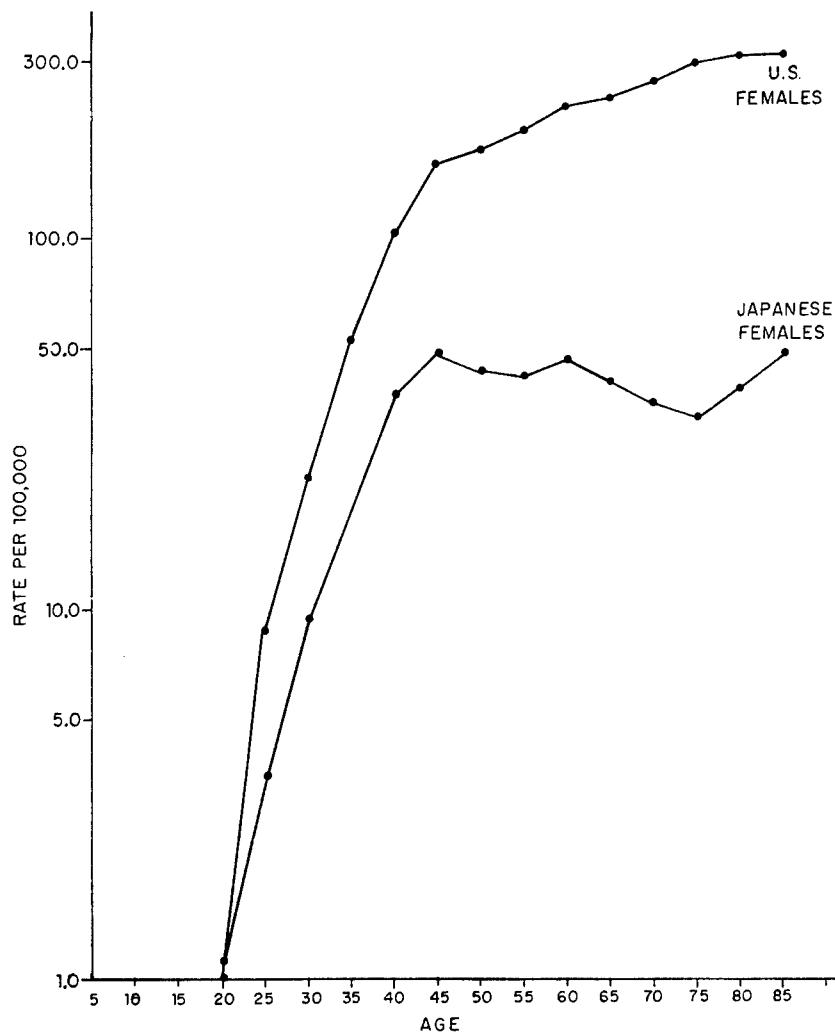


Figure 5. Age-specific incidence rates for female breast cancer, U. S. (1971) and Japan (1973). (From Segi et al., 1969).

The key difference in environmental factors between Japan and the Western countries such as the United States appears to be dietary in origin, with the main variable the quantity of dietary fat (Table 7). High risk populations consume a higher proportion of dietary fat, a percentage which has been increasing over the past fifty years (Gortner, 1975). This change may

be related to the slight but definite increased incidence of breast cancer in Western women over the same time span. DeWaard (1975) reported that obesity was a promotional factor in Dutch women, but this apparently is not seen in epidemiological studies on North American Caucasian women (Wynder et al., 1976).

TABLE 7. MEAN DIET VALUES, 24-HOUR RECALL

<u>Nutrient</u>	<u>Japan</u>	<u>Hawaii</u>
Calories	2,132	2,274
Total protein (g)	76	94
Animal protein (g)	40	71
Vegetable protein (g)	37	24
Total fat (g)	36	85
Principally saturated (g)	16	59
Principally unsaturated (g)	21	26
Total carbohydrate (g)	335	260
Simple (g)	61	92
Complex (g)	278	169
Alcohol (g)	28	13
Cholesterol (mg)	457	545

(From Stemmermann and Hayashi, 1977).

Migrant studies with respect to colon cancer have demonstrated that the first generation migrants from a low risk region have an appreciable increase in risk when transferred to a high incidence area such as the United States. However, in regard to breast cancer, first generation migrants show only a slight increase and it is not until the second generation that a risk similar to that of long-term residents in the high incidence region is attained. This aspect deserved further documentation since it suggests that, for breast cancer, residence in a high risk area at the time of puberty and breast development is critical. In a recent study of Japanese A-bomb survivors (pers. commun., Dr. C. Hand), it was found that irradiation between the ages of 10-19 resulted in an increased breast cancer incidence (15 years later) of approximately 5½% per radiation exposure above that of

unexposed women of the same age group. For colon cancer, where the cell kinetics are quite different, the factor of exposure at a young age is evidently not as critical. An analogous situation may be seen in the Huggins rat model. Female Sprague-Dawley rats are more sensitive to a single dose of hydrocarbon carcinogen at or around puberty than are older or younger animals and this susceptibility seems to be correlated with the rate of cell division and serum prolactin levels (Nagasawa and Yanai, 1975). The curve denoting sensitivity may be broader with the newly developed model of Gullino involving methylnitrosourea as a carcinogen (Gullino, Pettigrew and Grantham, 1975).

There have been a number of studies attempting to relate differences in breast cancer incidence between high and low risk populations to hormonal profiles in urine, serum, and plasma. Most studies have involved data on levels of urinary steroid hormones: estrogen and androgens (Hellman et al., 1971; MacMahon, Cole and Brown, 1973; Bulbrook et al., 1976). However, the etiological significance of abnormal or discriminating estrogen and androgen secretion patterns is a subject of controversy at present.

To investigate the relation of diet to the circulating hormone profile, we have compared a variety of ovarian and adrenal steroids in a low risk (Japanese) and high risk (North American Caucasian) population (Table 8). In premenopausal women, the main difference found was a higher level of estradiol in Japanese women in comparison to Caucasian women; however, in postmenopausal women, although estradiol levels were similar, the androstenedione and testosterone levels in Japanese women were significantly lower than their Caucasian counterparts. Although plasma steroid concentrations may serve as discriminant functions in Caucasian or Japanese populations, it remains to be determined whether a change in ovarian function in adrenal metabolism is causally associated with breast cancer. On the basis of our studies in animal models, we believe that while steroids are certainly important and relevant to the question of breast cancer growth and development, pituitary hormones and perhaps thyroid hormones merit greater attention than has been the case in the past.

TABLE 8. ESTROGEN AND ANDROGEN LEVELS IN HEALTHY
PRE- AND POSTMENOPAUSAL CAUCASIAN AND JAPANESE WOMEN

	<u>Estrone</u>	<u>Estradiol</u>	<u>Andro- stenedione</u>	<u>DHEA</u>	<u>Testo- sterone</u>
<u>Premenopausal</u>					
Caucasian (21)	24.6 \pm 2.5	23.8 \pm 1.0	285 \pm 21.6	352 \pm 25.4	45.4 \pm 3.5
Japanese (12)	30.4 \pm 3.3	29.8 \pm 1.9 ^a	358 \pm 44.3	294 \pm 41.9	49.0 \pm 5.1
<u>Postmenopausal</u>					
Caucasian (27)	24.4 \pm 3.5	7.7 \pm 0.6 ^b	219 \pm 15.5	230 \pm 26.5 ^b	40.5 \pm 1.8
Japanese (16)	22.3 \pm 2.3	5.9 \pm 0.8 ^b	90 \pm 6.1 ^a	301 \pm 34.1	28.0 \pm 1.5 ^{a,b}

^ap < 0.01 significantly different from Caucasian women.

^bp < 0.01 significantly lower in postmenopausal women.
(From Hill et al., 1976a).

Recently, emphasis has been placed on the role of prolactin in breast cancer (Smithline, Sherman and Kolodney, 1975; Horrobin, 1976). Although evidence in the rat model is convincing, evidence in humans is controversial and requires further substantiation. Elevated prolactin levels have been noted by Henderson et al. (1975) in the daughters of women with breast cancer in comparison with controls. Kwa et al. (1974) also reported a familial relationship between high serum prolactin levels and breast cancer. However, more recently Kwa et al. (1976) reported no relation between a variety of risk factors (age at 1st pregnancy, age at menarche) and circulating prolactin concentrations.

Since hypothalamapituitary (Wurtman and Fernstrom, 1975), adrenal (Savage, Forsyth and Carxeron, 1975) and ovarian activity (Fishman, Boyar and Helman, 1975) can be altered by dietary factors, we have attempted to modify this activity by transferring women from a standard Western diet to a low fat vegetarian diet. Initial data suggest not only that hormone levels such as testosterone and prolactin are altered, but that the menstrual cycle may be shortened by one to two days by dietary modification (Hill et al., 1976) (Figure 6).

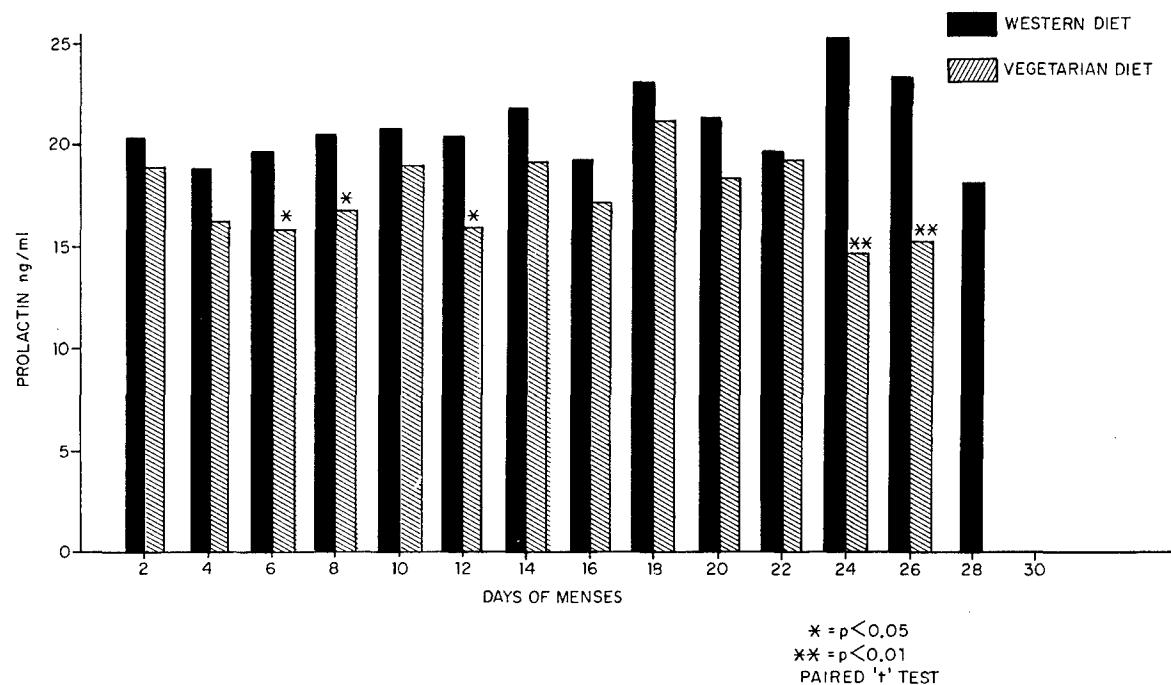


Figure 6. Prolactin levels in seven nurses taken on alternate days of the menstrual cycle. Nurses were maintained on a Western diet and transferred for two months to a vegetarian diet. Blood samples were taken on the Western diet and during the second month of the vegetarian diet. Differences between the prolactin levels were determined by a paired 't' test. * $p < 0.05$ ** $p < 0.01$. (From Wynder et al., 1976.)

In the same context, Hill and Wynder (1976) demonstrated that volunteers on a Western diet have higher prolactin levels than a similar group on a vegetarian diet, with the difference between the two diets being most pronounced during deep sleep (Figure 7). Accordingly, additional studies of 24-hour hormonal patterns in high and low risk populations are indicated in order to understand what specific hormonal changes may serve to stimulate breast carcinogenesis. The data obtained may also have diagnostic value to distinguish high risk individuals within a given population.

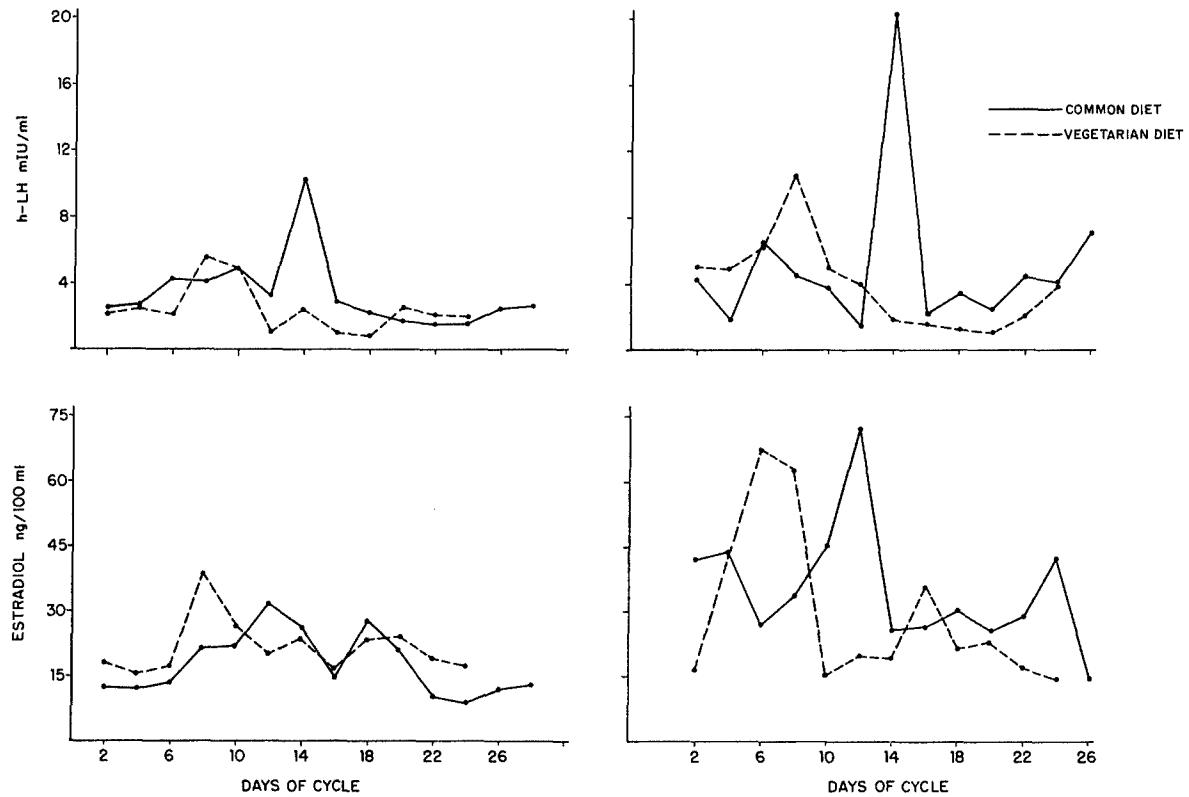


Figure 7. Nocturnal release of prolactin in four nurses maintained on a common Western diet and then transferred for two weeks to a vegetarian diet. Blood samples taken by an indwelling catheter. Sleep period began at 11 p.m. (From Hill and Wynder, 1976).

Animal Models of Breast Cancer

Experimental work conducted at our laboratory (Chan and Cohen, 1974) as well as that of others (Tannenbaum, 1942; Carroll, 1975) has shown unequivocally that a high fat diet promotes the development of both spontaneous and chemically-induced mammary tumors in rodents. The enhancing effect has been observed with many different types of fats and oils and is related more to the quantity of fat ingested than to the type of fat (Carroll and Khor, 1971). Nonetheless, at low (0.5%) fat levels, unsaturated oils appear to give a definitely higher tumor incidence on a per

animal basis than saturated fats. At high (20%) fat levels, the effect with both types is similarly high with the notable exception of coconut oil, which is unique in its low levels of linoleic and other C-18-20 fatty acids (Carroll, 1975). In contrast, the levels of protein or carbohydrate consumption did not significantly affect breast cancer development provided there was no dietary restriction (Tannenbaum, 1945).

By paired feeding of isocaloric high and low fat diets, Tannenbaum and Silverstone (1957) demonstrated that the high fat effect was independent of caloric intake. In addition, Carroll and Khor (1970) have shown that the high fat effect occurs only during the promotional phase of carcinogenesis, the most critical period being the first two weeks after initiation by a chemical carcinogen.

Thus, both epidemiological and experimental evidence suggest that the quantity of dietary fat is associated with breast cancer development.

Diet and nutrition apparently act as modifiers rather than initiators of tumor development. The modifying effect of diet may be exerted either directly or indirectly. Direct mechanisms involve specific alterations in cell membrane structure and functions; indirect mechanisms, on the other hand, involve changes in ongoing physiological processes. For example, diet can affect the endocrine system, colon bacteria and the substrates which colon bacteria metabolize, and possibly other systems, such as the mixed function oxidase system and the immune system. Recent reviews by Hopkins and West (1976) and Alcantara and Speckmann (1976) discuss in detail the current knowledge in this area.

In our laboratory, we have focused on two hormones, prolactin and estrogen. Both of these have been shown to play a critical role in the maintenance and continued development of breast cancer, prolactin possibly being the more important of the two (Pearson, 1973). Using the Huggins model, Chan and Cohen (1975) demonstrated that administration of the anti-prolactin drug 2-bromo- α -ergocryptine (Sandoz) to rats abolishes the tumor-promoting effect of a high-fat diet whereas administration of the anti-estrogen drug, U11,100A (Upjohn) did not eliminate the high fat effect. Further studies have shown that high fat intake elevates serum prolactin levels, particularly during the proestrous-estrous stage of the estrous cycle (Chan, Didato and Cohen, 1975) (Figure 8).

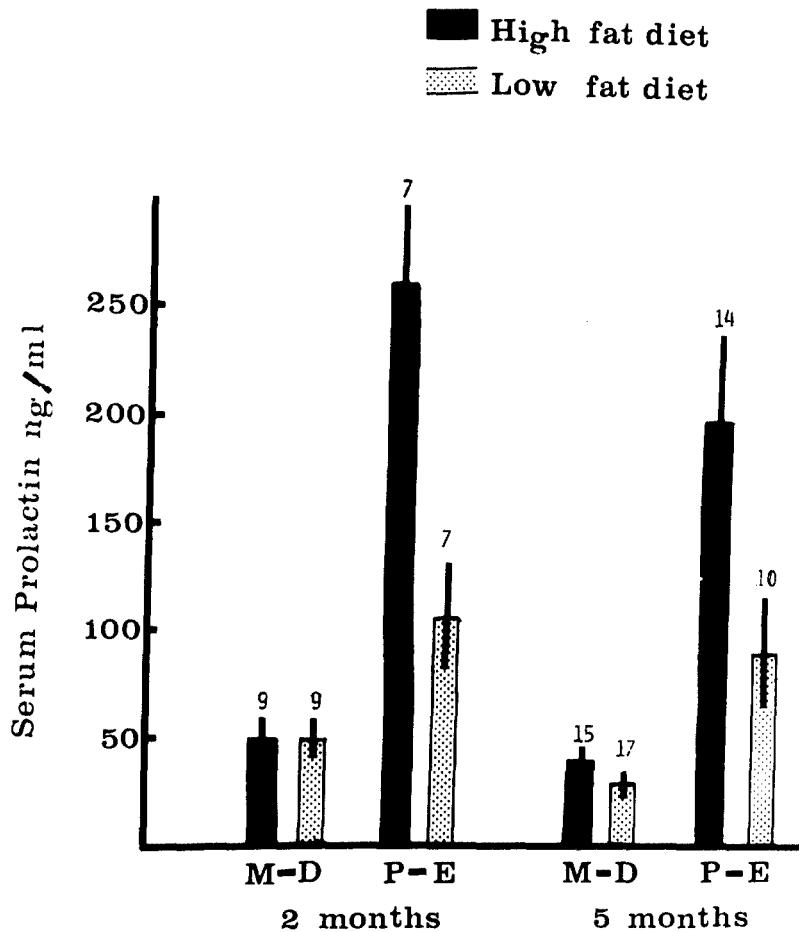


Figure 8. Serum prolactin titers of rats fed high (colored bars) and low fat (stippled) diets. Vertical line above each bar indicates \pm SEM. Numbers above lines designate the number of individual rats assayed at M-D (metestrus-diestrus) and P-E (proestrus-estrus). Difference between means at P-E was significant ($p<0.05$); that at M-D was not. (From Chan, Didato and Cohen, 1975.)

This study focused attention on two important methodological aspects of prolactin assays often neglected in human studies. Namely, that there is a distinct periodicity in circulating prolactin concentrations governed by (a) stage in the estrous (menstrual) cycle and (b) time of day (Horrobin, 1976). The

importance of distinguishing between basal and peak periods is exemplified in Hill and Wynder's (1976) finding that the most striking difference in prolactin levels in women on vegetarian or Western diets occurred during the late-night prolactin peak and not during the day.

Since prolactin is a well-known promoting agent in breast cancer (Furth, 1972; Meites, 1972; Pearson, 1972) Chan and Cohen (1975) proposed that the high fat effect was mediated by increased synthesis and/or secretion of prolactin by the pituitary gland. The resulting periodic hyper-prolactinemia induced by high fat intake was then envisaged to stimulate mammary tumor growth.

Results of in vitro studies on long-term cultures of normal and neoplastic mammary epithelium (Cohen, Tsuang and Chan, 1975) have added another element to this hypothesis. Estradiol above 1 $\mu\text{g}/\text{ml}$ was toxic to cultured mammary adenocarcinoma. Surprisingly, prolactin, at concentration ratios above 5-10 times that of estradiol, counteracted the inhibitory effect of estradiol (Figure 9). Though it remains to be seen whether this phenomenon can be explained within the present context of receptor theory (McGuire et al., 1974), it suggests that estrogen and prolactin act in opposition to one another at the level of the mammary tumor cell and that the actual quantitative relations between the two hormones may determine the rate of mammary cell metabolism and growth. No doubt this is an oversimplification, since other hormones such as progesterone, insulin, and hydrocortisone will eventually have to be integrated into this conceptual scheme (McGuire et al., 1974).

Precisely how prolactin acts at the subcellular level in either normal or neoplastic mammary epithelium is unknown at present. There is good evidence that cellular receptors for prolactin exist and are partially controlled by circulating prolactin concentrations (Kelly et al., 1974). Estrogens apparently can regulate prolactin receptor capacity as well (Vignon and Rochefort, 1974). Moreover, prolactin has been shown to induce changes in the cyclic AMP system principally at the level of the cAMP-activated protein kinase (Turkington, 1973) in normal mammary glands. We are currently studying the role of protein kinases in the action of prolactin on the growth and development of neoplastic mammary cells (Cohen, Tovbis and Chan, 1976).

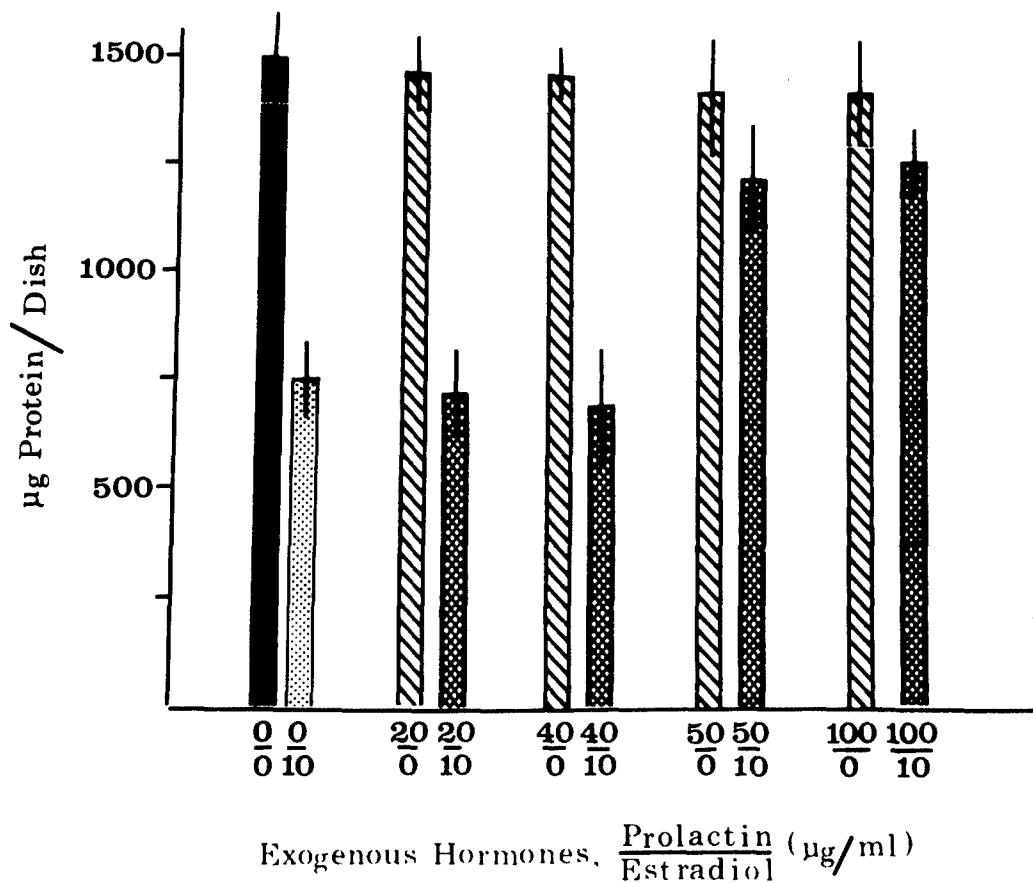


Figure 9. Effects of estradiol and prolactin alone (stippled and hatched bars) in combination (cross-hatched bars) on growth of rat mammary tumor cells. Colored bar represents growth in standard MEM + 10% fetal calf serum. Hormones were added at medium change on day 1, 4 and 7. Total protein was determined after 10 days growth. Lines above bars represent \pm SEM. (From Chan, Tsuang, Head and Cohen, 1976).

Recently, Gullino et al. (1975) described a method of inducing mammary adenocarcinoma in rats by multiple injections of the carcinogen methylnitrosomethylurea. The resultant mammary tumors exhibited many properties in common with the human disease: they exhibited metastasis, were hormone dependent, and were associated with hypercalcemia. Using this model, we have shown, as did Carroll (1975) in the DMBA model, that a high fat diet ingested after a single injection of NMU increased tumor incidence and decreased the latent period when compared to a similar population of rats fed a low fat diet (Chan, Cohen and

Wynder, 1976). In addition, assay of serum prolactin and estrogen levels in these animals revealed a significant increase in both prolactin and the prolactin/estrogen ratios (Table 9). Parallel studies in high and low risk human populations (Hill et al., 1976) have shown that Caucasian breast cancer patients have significantly higher mean plasma prolactin/estrogen ratios in comparison to their Japanese or Bantu counterparts.

TABLE 9. DIETARY FAT INFLUENCE ON SERUM HORMONAL PROFILES AND MAMMARY TUMOR INCIDENCE IN NMU-TREATED FISCHER RATS (GROUP 1)

Diet (Tumor Incidence)	Estrous Stage	Prolactin (ngs/ml)	Total Estrogen (ngs/100 ml)	Prolactin Estrogen ($\times 10^2$)
High Fat (90%)	P - E ^a	237 \pm 98 (8) ^b	23.7 \pm 1.7 (8)	11.1 \pm 4.8
	M - D	100 \pm 20 (7) ^c	22.2 \pm 2.0 (7)	4.5 \pm 0.9 ^d
Low Fat (40%)	P - E	140 \pm 79 (4)	23.2 \pm 2.2 (4)	6.4 \pm 3.9
	M - D	38 \pm 3 (12)	18.9 \pm 1.1 (12)	2.0 \pm 0.1

^aP - E: Proestrus-Estrus; M - D: Mestestrus-Diestrus.

^bMean \pm SEM, number in parenthesis indicates number of animals assayed.

^cp<0.05, high fat vs low fat.

^dp<0.01, high fat vs low fat.

(From Chan et al., unpublished results, 1977).

In summary, the study of animal models has provided new insights into the possible role of diet in the development of human breast cancer. A hypothesis based on the evidence from the rat model and partially verified in humans has been proposed which suggests that the tumor-enhancing effect of a high-fat diet may be mediated by an increased serum prolactin concentration, which is ultimately reflected in an increased prolactin to estrogen ratio in the circulation. Confirmation of this hypothesis in humans awaits analysis of the 24-hour profiles of prolactin in individuals from populations of high or low risk for breast cancer.

PROSTATE CANCER

Epidemiology has provided data on the incidence of prostate cancer as a function of a number of factors such as race, age, area of residence and diet (Wynder, Mabuchi and Whitmore, 1971). Thus, Caucasians in the Western world have a higher incidence of prostatic cancer than Japanese. First generation Japanese migrants to the United States retain their low risk but later generations exhibit a higher risk (Akazaki and Stemmerman, 1973). However, native Japanese (Akazaki, 1973) have *in situ* lesions, and it has been suggested that the difference in proliferative types of prostatic carcinoma as seen in Western men is due to promotion by environmental factors, of which the diet deserves primary consideration.

In the United States, blacks, particularly in the South, have a lower risk for both colon and breast cancer, and these diseases are increasing at the present time, presumably because of sizeable alterations of dietary habits in the South. However, the risk for prostate cancer is higher in American blacks than in American whites. This factual observation has not yet received any explanation or even rational hypothesis. Nonetheless, it is clear that as a rule, population groups who have high risk of breast, colon, and endometrial cancer also have a high risk of prostate cancer (Armstrong and Doll, 1975). Inasmuch as for the former, an association with diet has been documented not only in population studies and epidemiology, but also in metabolic and physiological approaches, it is probable that dietary fat plays a role in the etiology of prostate cancer. For breast and colon cancer, the dietary fat hypothesis is further supported by detailed studies in animal models as described above. Unfortunately, at present, there is no reliable and realistic animal model for prostate cancer, amenable to mechanistic studies.

Dietary factors may become operative during puberty, or at some later time. Physiological development of boys from different socio-economic groups in Hong Kong was greater in higher socio-economic groups (Lee, Chang and Chan, 1963). Trends in increasing stature of Polish boys over the last eighty years further suggest dietary factors are involved (Wolanski, 1966). The effect of diet on growth, height, and weight is also evident in Japanese boys (Frisch and Reveille, 1969). From 1948 to 1963, the total protein intake increased 12% from 63 to 70.6 g per day, but the animal protein rose 113%, from 11 to 27.9, while the age of maximum height

increment decreased from 14.5 to 12.5. The animal protein component also included animal fat. Comparable data for American-born Japanese boys for the maximum increase in height is 10.5 years. Although the data clearly implicated nutritional factors in stimulating puberty and growth, the effect of nutritional factors, including protein, fat and micronutrients, on the endocrine patterns in boys and men in relation to prostate cancer risk remains to be clarified.

Since 1890, it has been known that castration produces prostatic atrophy. The relationship of castration and sex hormones to histological changes in prostatic tissue was established by Bandes (1966), who demonstrated that testosterone promoted the growth of prostatic epithelium, while estrogen and castration reversed or inhibited epithelial cell growth.

Although this disease occurs predominantly in men over 50 years of age, little decrease in the plasma testosterone level occurs until late in life (Kent and Accone, 1966), nor is there any significant decrease in prostatic cancer patients (Sciarra et al., 1973).

We are studying the relationship between diet and urban and rural Polish, Bantu, white South African, and American white and black men at various ages and examining several gonadal, adrenal and pituitary hormones.

CONCLUSION

We have presented an overview of the concepts that the etiology of the main human cancers stem largely from our lifestyle. The underlying mechanisms and supporting research development have been discussed. Thus, most cancers of the respiratory tract are due to excessive smoking. Prevention of these cancers hinges on educating the public about the hazards connected with this habit, not only with respect to the development of cancer of the lung, pancreas, kidneys and bladders, but also with respect to myocardial infarction. In addition, those who cannot be induced to stop their cigarette habit should be convinced to smoke the lower tar cigarettes now available, since evidence is showing that these indeed have a lower risk. Further, through managerial approaches to prevention, industry and government should collaborate further toward the production and preferential marketing of such lower risk cigarettes.

Cancers of the endocrine-controlled organs - breast, prostate, ovary and endometrium are associated, in large measure, with diet and more specifically, with the high fat content of the diet. These concepts are underwritten not only by human data but also by detailed studies with animal models for colon and breast cancer, in which approaches to the underlying mechanisms could be formulated. In addition, cancer of the pancreas is also associated, in part, with such dietary customs.

Prevention of these cancers hinges on the development of altered dietary customs to lower fat consumption from the now prevailing level of 45% of calories. One step in the right direction is the adoption of the "Prudent Diet", with about 33% of all calories as fat and less than 100 mg of cholesterol, recommended for the prevention of heart disease (Bennett and Simon, 1973). However, to reduce the cancer risk, it may be necessary to lower the fat content further to 25-27% of all calories. The Connors (1972) recommended a level of only 20% of calories. Especially with respect to intestinal cancer, but perhaps including the other diet-related cancers, the question of dietary fiber, or micronutrients such as Vitamins A and E, or minerals, such as selenium, requires consideration and further research.

The approach involving managerial prevention would have industry, in cooperation with agricultural and governmental agencies, produce and market foodstuffs which would permit the consumer to adopt the Prudent or even the lower fat diet more readily and, in fact, automatically.

Cancer of the stomach, already on the decline in the United States, is still the major cancer in Western Latin America, the Orient, and Northern and Eastern Europe. This cancer may be prevented by increasing the consumption of foods high in Vitamin C, through low temperature food storage or prevent nitrite accumulation and through the controlled use of nitrite as a preservative.

If these measures are taken and if, in addition, the readily preventable occupational cancers are eliminated, we would enter an era where cancers of all types would no longer represent a major cause of death in man.

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SESSION I

INDUSTRIAL TOXICOLOGY

Chairman

James H. Sterner, M.D.
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EXPERIMENTAL ONCOGENESIS IN RATS AND MICE EXPOSED TO
COAL TAR AEROSOLS

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Toxic Hazards Research Unit
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Dayton, Ohio

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6570 Aerospace Medical Research Laboratory
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and

Lester D. Scheel*

The National Institute for Occupational Safety and Health (NIOSH) prepared a criteria document entitled "Occupational Exposure to Coke Oven Emissions" which was published in 1973. This document, unlike other NIOSH criteria documents, did not recommend exposure limits but was published as a "work practices" document. In the preface to that document is the statement: "Due to the absence of reliable dose response data, this report does not recommend an environmental air standard as a safe exposure level". The absence of dose response data led to a series of studies, the latest of which is reported herein.

Percival Pott (1775) was first to recognize the relationship between scrotal cancer and the occupation of chimney sweeps. The earliest association between these scrotal cancers and coal distillation products came from the writings of Volkmann in 1875 and Butlin (1892). These observations ultimately led to the inclusion of skin cancer resulting from the handling of pitch, tar and tarry compounds as a compensative disease under the British Workman's Compensation Act of 1907.

*Deceased, formerly with NIOSH, Cincinnati, Ohio.

Evidence that coal tar could also cause lung cancer was first reported by Kennaway and Kennaway (1947), who described the incidence of excess lung cancer mortality in British gas stokers and coke oven workers. A follow-up to this study was reported by Reid and Buck (1956). They conducted a survey of the occupational histories of all men who died between 1949 and 1954 after working in British coking plants and found that the number of deaths from cancer in the coking industry was comparable to cancer mortality experience in other large industries. This observation obscured the importance of the findings of the Kennaways until Lloyd (1971) found that the suspected increase in lung cancer in coke oven workers was limited to men employed at the ovens. The relative mortality for this disease was $2\frac{1}{2}$ times that predicted. The greatest part of this excess was accounted for by an almost 5-fold increase in the risk of lung cancer for men working on the tops of coke ovens and a 10-fold greater risk was observed for men employed 5 or more years at fulltime topside jobs. Fifteen lung cancer deaths were observed among the 132 men in this group compared with 1.5 deaths expected.

Redmond et al. (1972) have increased the numbers of coke oven workers from the study of Lloyd and have verified those findings.

Although coal tar has been shown experimentally to produce skin tumors in animals (Friedewald and Rous, 1944), its ability to produce lung tumors in experimental animals has not been adequately demonstrated. Horton et al. (1963) exposed C3H mice to massive air concentrations of coal tar aerosol from which the solids had been removed. These exposures, conducted three times weekly for 2 hours each time at an exposure concentration of 300 mg/m³, killed all of the animals after 35 weeks. Five of 33 mice examined histologically had squamous cell lung tumors, one of which was described as a carcinoma. A control group of C3H mice kept long beyond the termination of the exposure group did not show any similar tumorous lesions.

We conducted a series of 90-day continuous inhalation exposures of rats, mice, and rabbits to coal tar aerosol at 20, 10, 2, and 0.2 mg/m³ air concentrations. Although complete histopathologic data have not yet been made available, significant dose related effects on growth were observed as was a dose dependent incidence of skin tumors in mice. In order to compare these findings with the results of long-term chronic industrial type exposures, an 18-month study was designed.

METHODS

Two large inhalation chambers, previously described by Thomas (1968), housed the experimental animals used in this study. The animals were distributed between the two chambers as shown in Table 1. Equal numbers of control animals were held in our vivarium for comparative purposes. Aerosol exposures were conducted each regular working day for a 6-hour period to simulate industrial working conditions. The exposures were conducted for 18 months, after which the exposed animals were removed from the chambers and returned to the vivarium and held for an additional 6 months of observation prior to necropsy.

TABLE 1. EXPOSURE CHAMBER LOADINGS FOR COAL TAR AEROSOL STUDY

Chamber 1

18 New Zealand Albino Rabbits (Female)
40 Sprague-Dawley Weanling Rats (Male)
40 Sprague-Dawley Weanling Rats (Female)

Chamber 2

5 Macaca mulatta Monkeys (Male)
9 Macaca mulatta Monkeys (Female)
75 ICR CF-1 Mice - Group A (Female)
100 ICR CF-1 Mice - Group B (Female), for serial sacrifice
50 CAF₁-JAX Mice (Female)

The coal tar used to generate the aerosol in this study was a composite mixture collected from multiple coking ovens around the greater Pittsburgh area. The coking ovens were of several different types and used different coal sources for their starting materials. The coke oven effluents were collected in air collection devices using a chilled water spray to condense the higher boiling distillate fractions. After settling and separation of the liquid phase, the various coal tar samples were blended together with a 20% by volume amount of the BTX (benzene, toluene, xylene) fraction of the coke oven distillate. This ratio of coal tar to BTX fraction is that usually found in coke ovens operating in the 800-1100 C range. The recombination of the coal tar and BTX fractions not only made the resultant material more like that inhaled by topside coke oven workers but was a critical factor in the generation of the aerosol. The BTX functioned to decrease viscosity or reduce solids, permitting aerosolization.

Because the coal tar-BTX mixture was extremely viscous and contained some fine particulate material, most aerosol generation methods such as ultrasonic nebulization were unusable, resulting in phase separation. A technique was developed to feed the coal tar mixture under slight pressure into a 1/8" stainless steel cross piece meeting a high pressure (35 psi) air stream as shown in Figure 1. Animal exposure chamber concentrations were regulated by altering the speed of the polystaltic pump.

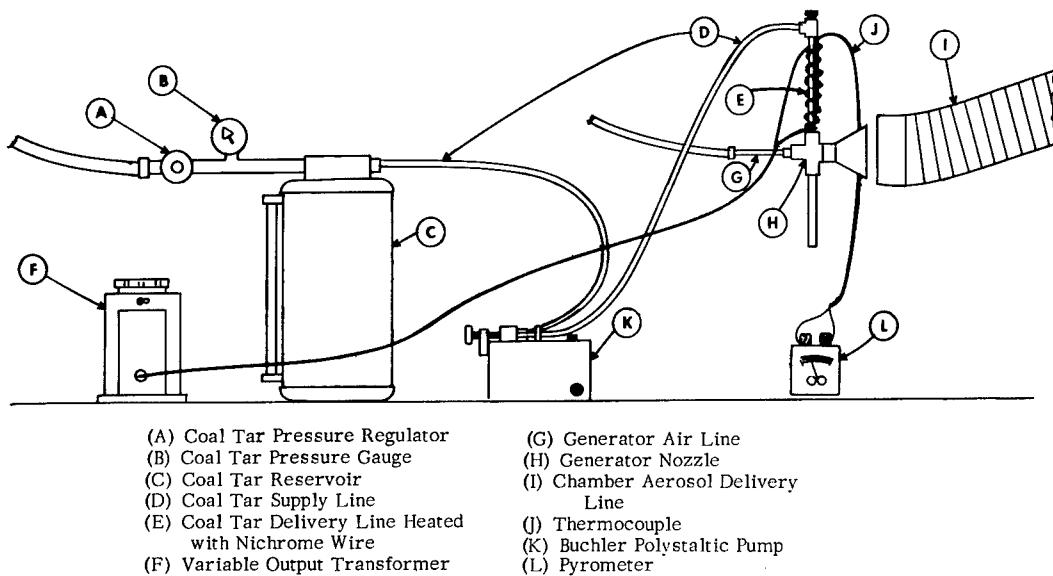


Figure 1. Contaminant generation system for aerosolization of coal tar volatiles.

The chamber concentrations were mounted using a combination of a hydrocarbon analyzer and a fluorometer. In the latter system, gravimetric sampling techniques were used to collect the aerosol droplets on cellulose acetate filters from which the fluorescent materials were dissolved in toluene and measured against calibration curves made with weighed amounts of the coal tar mixture. The chamber concentrations were monitored on an hourly basis with this method. A hydrocarbon analyzer was calibrated against the fluorometric measurements. The hydrocarbon analyzer was then used as a continuous monitor of chamber concentrations but was rechecked periodically since the vapor-droplet ratio could change slightly with time or with different barrels of the coal tar mixture.

Aerosol particle size determinations were made monthly during the study using the procedure of Vooren and Meyer (1971). A minimum of 99% of the total droplets in both chambers were 5 microns or less in diameter. Thus, the majority of particles were within the respirable size range for rodents. Other standard methods for determination of particle size were impractical for routine use because of the semi-liquid nature of the coal tar aerosol which might coalesce on surfaces and plug or damage complex instrumentation.

The weanling rats used in this study were the Sprague-Dawley derived CFE strain while mice of two strains were used. A tumor susceptible strain (ICR CF-1) was obtained from Carworth Farms, and a tumor resistant hybrid strain (CAF₁-JAX) was obtained from Jackson Laboratories. The ICR CF-1 mice were divided into two groups by a random selection process and one group of 75 animals was held throughout the study for skin tumor incidence evaluation and pathology examination only. The second group of 100 mice was serially sacrificed during the exposure period for determination of the coal tar lung burden and an attempt to determine the time to tumor induction. The latter group of mice was not included in tumor incidence evaluation due to statistical problems arising from their usage, although those that were necropsied in the second year of the experiment appeared to have the same tumor incidence as seen in the other group.

All experimental and control animals were examined daily during the exposure and postexposure periods. Rats and monkeys were weighed biweekly for the first four months of the study and on a monthly basis thereafter. The rabbits were weighed on a similar schedule until the ninth month when an insufficient number of surviving rabbits made further comparison with control group weights useless.

RESULTS

The results of growth rate observations made on the coal tar exposed and control rats are shown in Figure 2. The difference between control and exposed animals was statistically significant by the fourth month for both male and female rats and remained significant throughout the remainder of the study.

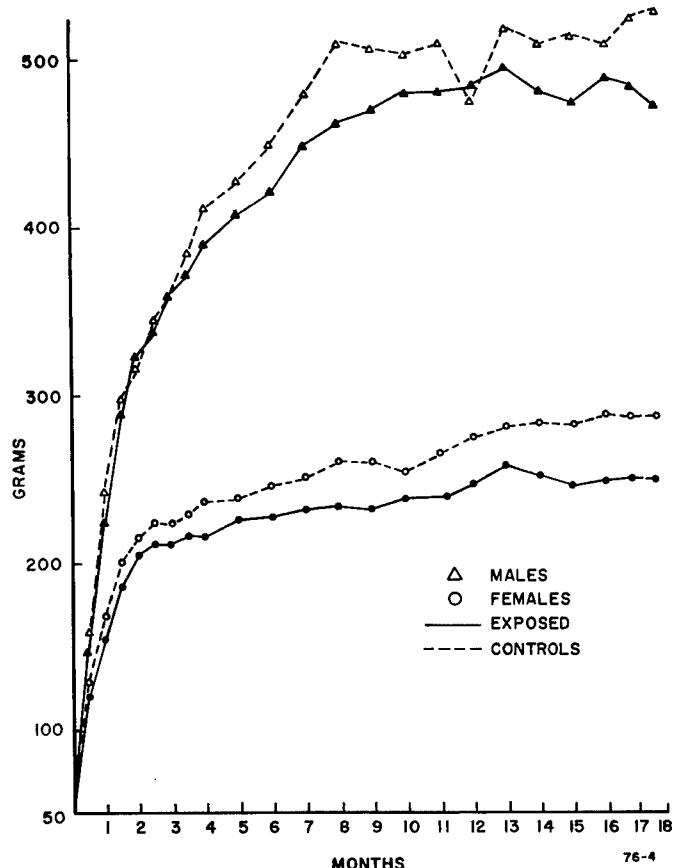


Figure 2. The effect of repeated exposure to 10 mg/m³ coal tar aerosol on growth of rats.

Monkey and rabbit weights are shown in Figure 3, where it can be seen that there were no significant effects on growth in the monkeys exposed to the coal tar aerosol. Rabbits exhibited a significant difference by the end of the first month. Sixteen test and 6 control rabbits died during the exposure period; this mortality has been attributed to a chronic respiratory infection which caused debilitation and dehydration. At the conclusion of the 18-month exposure period, the monkeys and the surviving rabbits along with their unexposed controls were delivered to the NIOSH Laboratories in Cincinnati, Ohio for long-term postexposure observation; therefore, no further comment on these species will be made in this presentation.

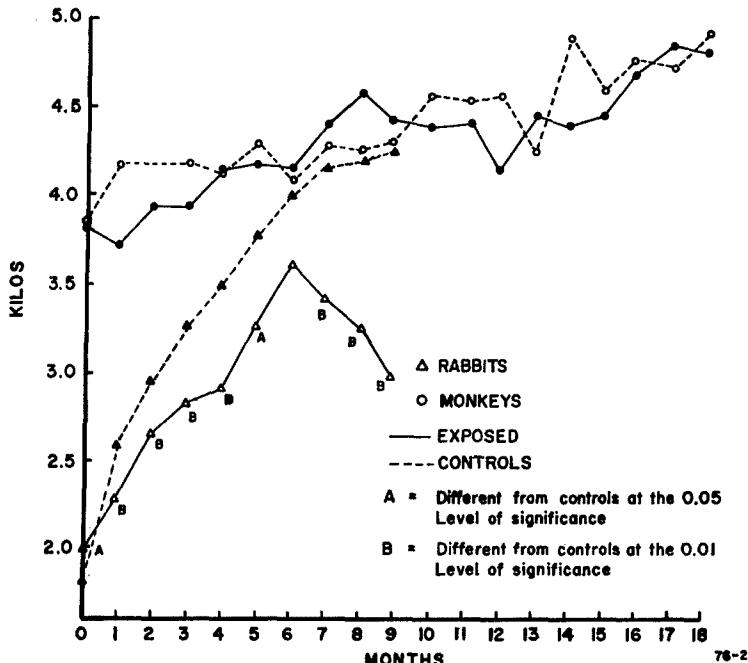


Figure 3. The effect of repeated exposure to 10 mg/m^3 coal tar aerosol on growth of rabbits and monkeys.

Skin tumor incidence (Table 2) seen in both strains of mice exposed to the coal tar aerosol 90-days continuously and held 21 months postexposure demonstrated a dose response relationship. Although not apparent in the table, the occurrence of these skin tumors was delayed until after termination of the exposure regimen and, in the ICR CF-1 strain mice, continued to develop for as long as 86 weeks postexposure even though residual coal tar appeared to have been completely removed from the skin shortly after completion of exposures.

TABLE 2. SKIN TUMOR INCIDENCE IN MICE CONTINUOUSLY EXPOSED TO A COAL TAR AEROSOL-BTX MIXTURE FOR 90 DAYS

Exposure Group	ICR CF-1 Mice Incidence	ICR CF-1 Mice Percentage	CAF ₁ -JAX Mice Incidence	CAF ₁ -JAX Mice Percentage
10 mg/m^3	44/55	80	18/43	42
2 mg/m^3	14/75	19	3/65	5
0.2 mg/m^3	1/61	2	0/75	0
Control	3/225	1	0/225	0

Our criterion for counting a lesion as a skin tumor was a growth greater than 1 mm in diameter and in height but most tumors continued to grow as shown in Figures 4 and 5. Each tumor was ultimately confirmed by histologic examination.



Figure 4. ICR CF-1 mouse with horn-like growths on ear and eye.



Figure 5. ICR CF-1 mouse showing keratinaceous tumors at various body sites.

A sharp contrast in skin tumor incidence was seen (Table 3) when the same 10 mg/m³ coal tar aerosol concentration was presented to mice in an intermittent industrial type of exposure. A calculation of total exposure time reveals that the same amount of coal tar aerosol reached the skin of the two experimental groups, but the 18-month intermittent exposure group had time each day to permit normal cleaning of the fur.

TABLE 3. SKIN TUMOR INCIDENCE IN MICE EXPOSED TO
10 MG/M³ COAL TAR AEROSOL CONTINUOUSLY FOR 90 DAYS OR
6 HOURS DAILY FOR 18 MONTHS

	<u>ICR CF-1 Mice</u>	<u>CAF₁/JAX Mice</u>
90-Day Continuous Control	44/55 3/225	18/43 0/225
18-Months Intermittent Control	5/75 3/75	2/50 1/50

Measurements of hide and lung fluorescence are presented in Table 4. The amount of coal tar found on mouse skin did not change to any great degree after the first week of exposure. Lung tissue accumulated coal tar aerosol at a fairly steady rate during the entire exposure period of 18 months. The coal tar lung burden in mice is depicted in Figure 6 for both intermittent and continuous exposure. The area under the curves is approximately equal for both exposure modes, differing only in duration of the pulmonary tissue challenge.

TABLE 4. COAL TAR FLUORESCENCE RETAINED IN MOUSE LUNG AND SKIN TISSUES (N = 4)

<u>Days of Exposure</u>	<u>Calendar Days</u>	<u>Tissue Fluorescence*</u>	
		<u>Lung (μg/g)</u>	<u>Hide (μg/cm²)</u>
1	1	6	1.4
7	9	19	3.2
20	25	173	4.9
60	88	180	3.8
108	147	210	3.7
149	210	236	2.7
185	266	332	3.5
233	329	315	3.2
270	385	296	2.1
309	448	383	6.7
352	511	567	3.8
371	542	584	6.3

*Values are test animal values minus control animal values.

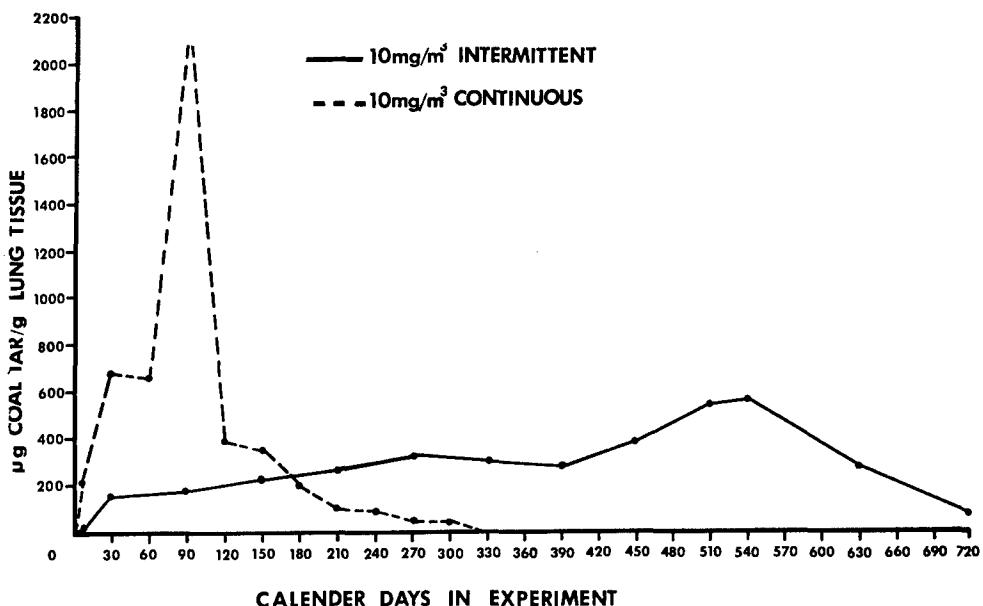


Figure 6. A comparison of total lung burden of fluorescent coal tar compounds between 90-day continuous and 18-month intermittent exposure.

The tumor incidence in rats exposed to the coal tar aerosol is presented in Table 5. A few miscellaneous tumor types were seen in female unexposed control rats but none were seen in males. The significant finding in these animals was the presence of squamous cell carcinoma of the lung in 100% of the male and 82% of the female rats. These tumors were readily seen at necropsy as shown in Figure 7 and under microscopic examination (Figure 8) where the typical keratinization of these tumors appears.

TABLE 5. COAL TAR TUMORIGENESIS IN RATS

	Controls		Exposed	
	Males	Females	Males	Females
Number Examined Histologically*	36	37	38	38
Tumors Found:				
Squamous Cell Carcinoma, Lung	0	0	38	31
Sebaceous Cell Carcinoma	0	1	0	0
Intraabdominal Carcinoma	0	1	0	0
Mammary Fibroadenoma	0	1	0	3
Mammary Adenocarcinoma	0	1	0	0
Other Tumors	0	1	8	2
Overall Tumor Incidence (%)	0	13	100	82

*The original number of rats per group was 40. However, because of autolysis and/or cannibalization, a few animals were unsuited for histopathological examinations.

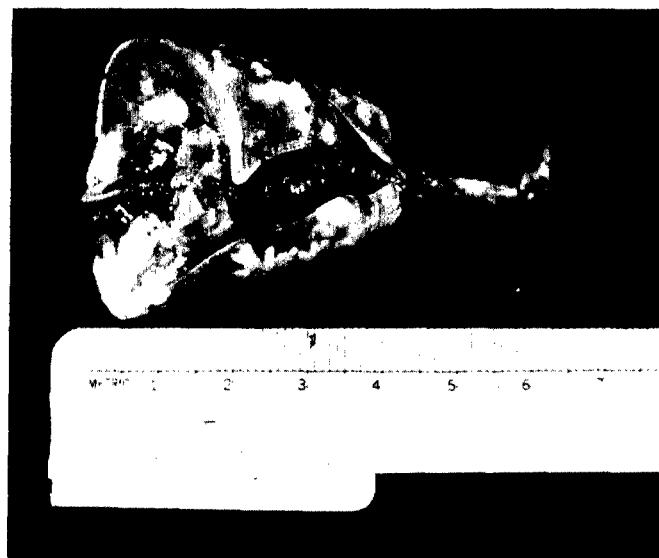


Figure 7. Gross appearance of coal tar aerosol induced tumors in rat lung.

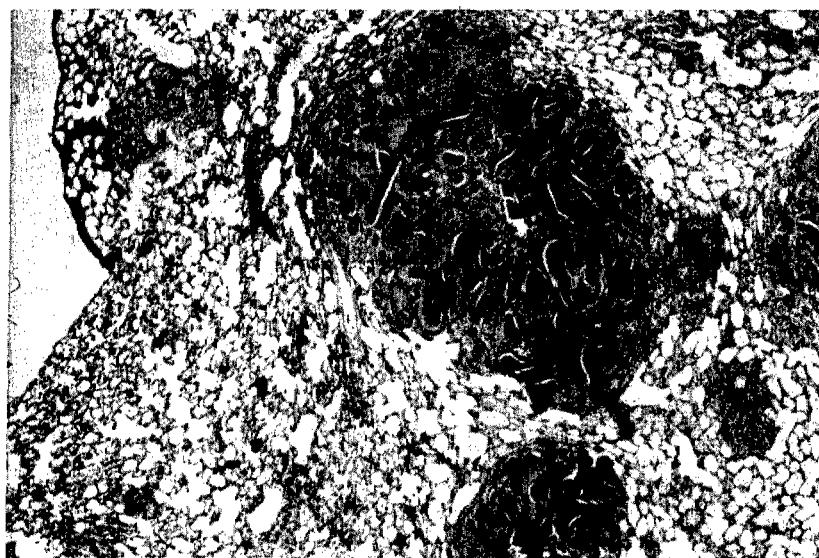


Figure 8. Low power magnification of coal tar induced tumor in rat lung showing keratinization.

The incidence of all tumor types seen in unexposed control and coal tar aerosol exposed mice is tabulated for each strain in Table 6. A large variety of spontaneously occurring tumors was observed in control mice of both strains. A comparable incidence of most of these tumors was seen in the mice exposed to 10 mg/m³ coal tar aerosol for 18 months with the notable exception of alveolargenic carcinomas where a significantly increased incidence was observed for both mouse strains. Alveolargenic adenoma was the most common form of spontaneous tumor and is sometimes identified as a carcinoma. To illustrate the difference, Figure 9 depicts the discrete edge of the tumor and compressed lung surrounding the tumor. In contrast, the infiltrative nature of an alveolargenic carcinoma in a mouse lung is shown in Figure 10.

TABLE 6. COAL TAR TUMORIGENESIS IN MICE

	Controls		Exposed	
	ICR/CF-1	CAF ¹ /JAX	ICR/CF-1	CAF ¹ /JAX
Number Examined Histologically	68	48	61	50
Tumors Found:				
Alveolargenic Carcinoma	3	8	26	27
Alveolargenic Adenoma	13	11	13	13
Bronchogenic Carcinoma	1	0	1	1
Squamous Cell Carcinoma	3	1	5	2
Lymphosarcoma	5	3	6	2
Reticulum Cell Sarcoma	4	4	5	7
Hemangiosarcoma	3	1	3	0
Hemopoietic Tumors	13	1	7	0
Subcutaneous Sarcoma	1	0	2	0
Other Tumors	3	5	3	6

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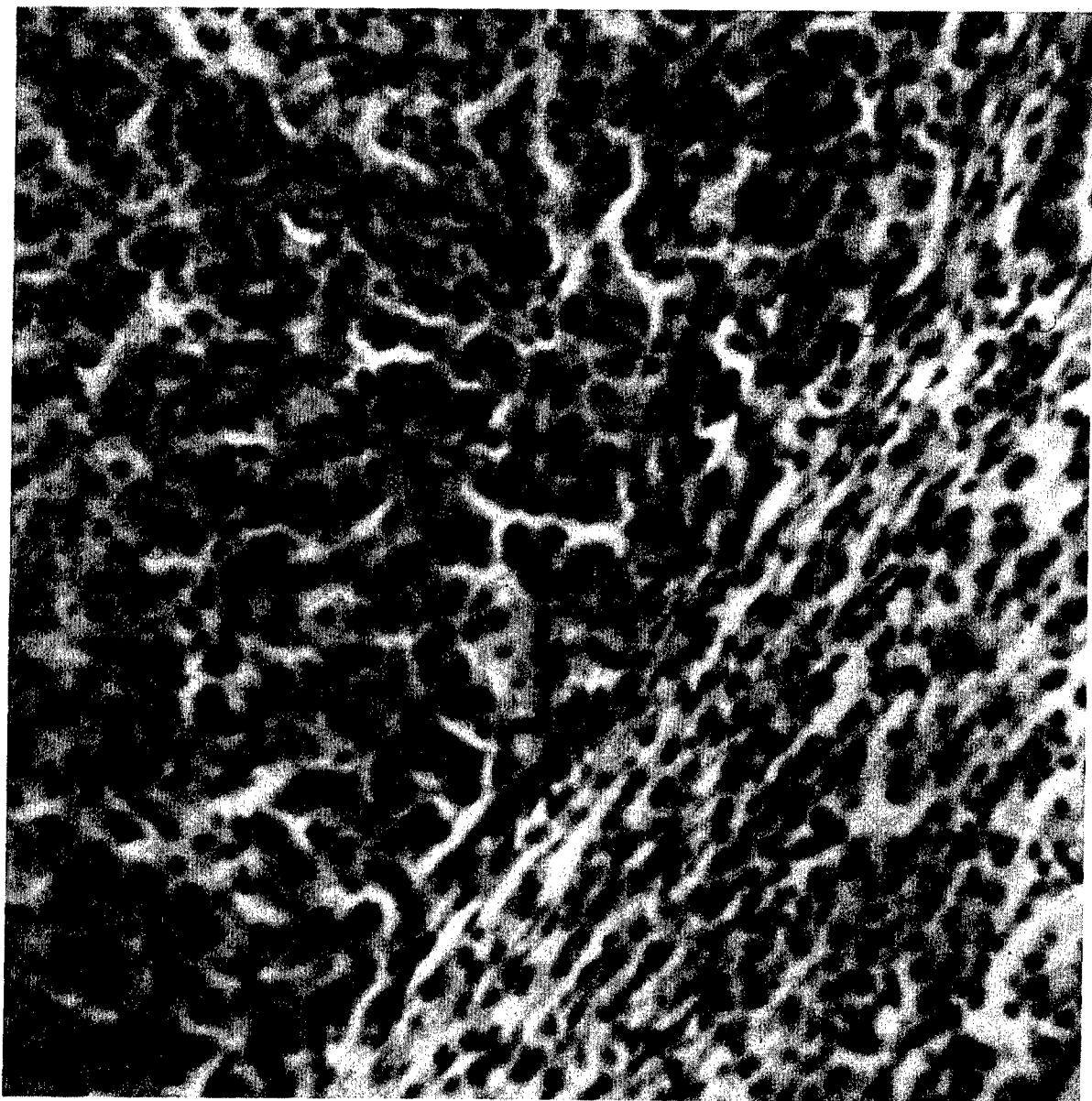


Figure 9. Alveolargenic adenoma in mouse lung tissue.
Approximate 1500X.

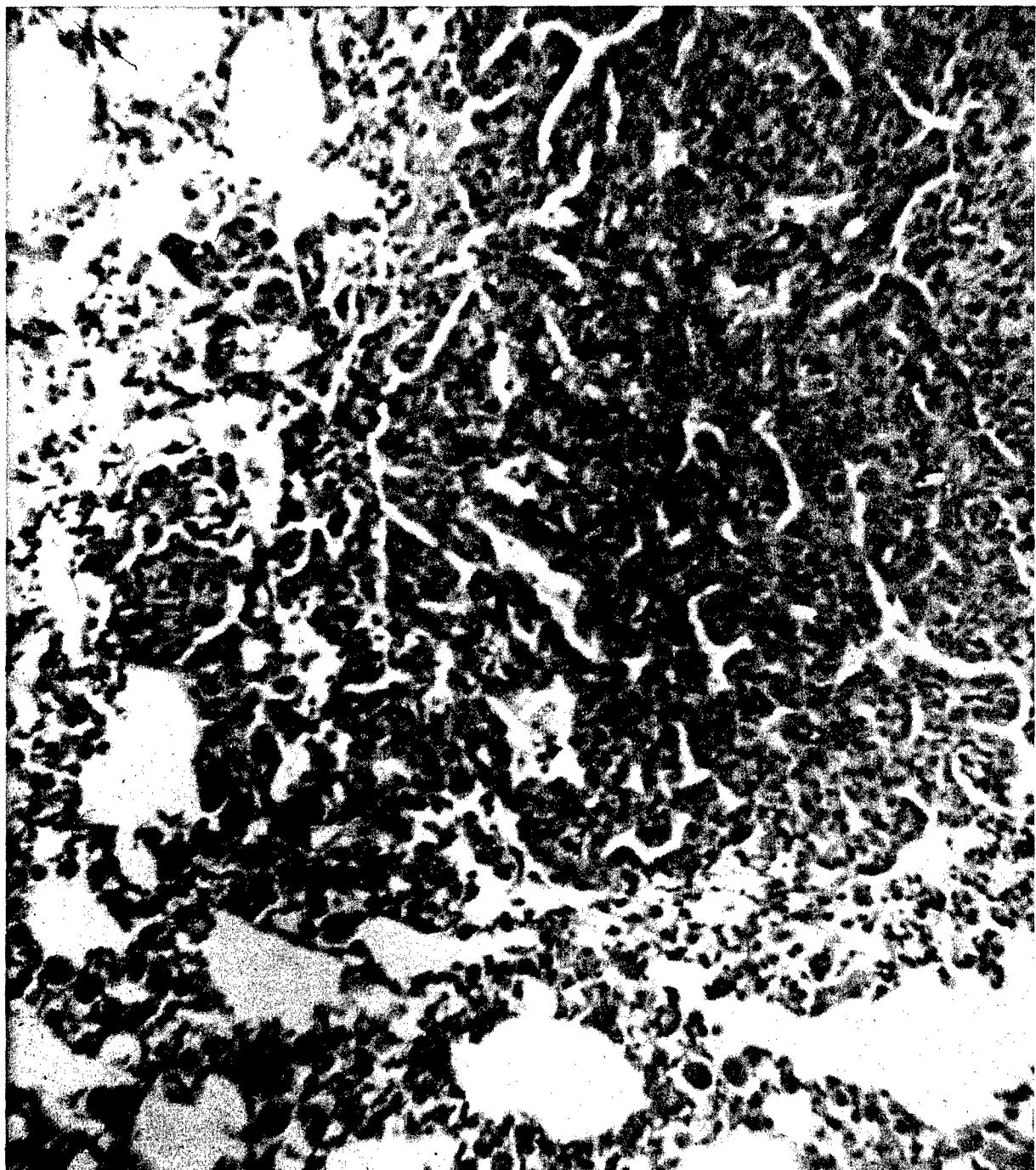


Figure 10. Alveolargenic carcinoma in mouse lung tissue.
Approximate 500X.

SUMMARY

This study represents a demonstration of an animal model of the increased lung tumor incidence seen in coke oven workers. Both rats and mice exposed to coal tar aerosols at air concentrations not unlike those seen on the top of coke ovens had significant increases in pulmonary carcinomas. Since the ability of coal tar to produce skin tumors in two mouse strains is dose dependent, it is probable that a similar dose dependency occurs with pulmonary tumor induction. This concept appears to be substantiated by the differences seen in cancer incidence and risk of workmen exposed to coal tar aerosols at various locations around the coking ovens.

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A HEALTH EFFECTS STUDY IN COKE OVEN WORKERS

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Our understanding of the biological effects of toxic exposures has come both from controlled animal studies and from observations on humans. In animal studies one can observe all gradations of exposure given for varying durations to groups of genetically distinct animals. In studying human exposures, these and other attributes of a closely controlled study are rarely possible because of practical as well as moral and ethical considerations. Moreover, the confounding influences of each person's varying genetic background, the complex and often unknown other environmental exposures, and other factors severely limit the investigator's ability to establish specific causal relationships. Despite these drawbacks, important findings have resulted from studies of accidental, acute human exposures to high concentrations of toxic substances as well as of chronic, relatively low-dose exposures. Of these, the most extensively documented, "voluntary," chronic, human toxic exposure affecting the lungs involve cigarette smoke, which is implicated in the majority of cases of human lung cancer and chronic obstructive lung disease. However, smoking neither accounts for all such cases nor, obviously, do all smokers suffer from such illnesses. Because of this and because the role of cigarette smoking has been virtually exhausted as a productive area for uncovering new knowledge about the causal relationship between toxic inhalation exposures and disease, much scientific attention is now increasingly turning to the workplace. Studies of the relationship between toxic exposures in various work environments and the subsequent development of changes in respiratory structure and function offer an opportunity not only to advance biological knowledge but to alleviate suffering through disease prevention.

Working conditions involved in the production of metallurgical coke have long attracted attention as a possible cause of excess mortality and morbidity. The classical studies of Lloyd, Redmond and others, reviewed by Dr. Redmond in this conference, clearly established both that coke oven workers are at excess risk of dying from cancers of the lung and genitourinary tract, and that the risk increases with the extent and duration of exposure. An excess risk of mortality or morbidity from other respiratory diseases has been suggested but not clearly established. Lowe et al. (1969) reported that symptoms were more common in those steel workers (including coke oven workers) most exposed to dust and sulfur dioxide. Ventilatory function was also significantly impaired in exposed workers. However, these authors commented that in comparison with the influence of cigarette smoking on symptoms and lung function, the "work relationship is trivial." Walker et al. (1971) reported that a relationship between work factors and symptoms could be clearly seen only in coke oven workers who smoked. As in studies in other industries such as mining (Sluis-Cremer et al., 1967), this observation suggested that cigarette smoke acts in synergism with the occupational exposure. Ventilatory function was significantly lower in workers exposed to the coke oven atmosphere as compared to workers in other areas, findings in keeping with earlier observations reported by Higgins et al. (1959). Statistical analyses carried out by Walker suggested that the presence or absence of symptoms of bronchitis had a greater influence on ventilatory function than did occupation.

Since, to our knowledge, morbidity studies had not been systematically performed on coke oven workers employed in the United States, we initiated a project at a local integrated steel mill several years ago. In an earlier report (Mittman et al., 1974) we described this study and pointed out that in a group of over 240 workers employed on a coke oven for over five years, the severity of symptoms of bronchitis could be related to their cigarette exposure, work histories and to possible genetic factors. As an extension of that study we are now annually testing these workers and others in selected areas of employment at this steel mill. This paper reviews preliminary findings of the first set of tests of respiratory structure and function in these steelworkers.

METHODS AND RESULTS

QUESTIONNAIRE AND WORKER CHARACTERISTICS

All workers in selected departments of the mill were asked to volunteer for these studies; more than 85% of those asked consented. All were men. Each worker completed a detailed self-administered questionnaire which yielded information about current and past jobs, respiratory symptoms, smoking histories, and other health information (Mittman et al., 1974). This questionnaire was a simplified version of the British Medical Research Council Respiratory Disease Questionnaire to which was added sections on occupational history, family history and other features of the worker's background. Table 1 summarizes the characteristics of the 699 workers who are included in the present analysis.

TABLE 1. CHARACTERISTICS OF THE WORKERS STUDIED,
DIVIDED INTO WORK-SITE GROUPS

<u>Work Area</u>	<u>N</u>	<u>AGE</u>	<u>YEARS WORKED</u>	<u>% SMOKERS</u>	<u>% EX-SMOKERS</u>
Crane	236	40.7 \pm 12	12.2 \pm 10	47(27 \pm 22)	25(23 \pm 24)
Masonry	168	36.8 \pm 13	10.6 \pm 10	41(17 \pm 16)	15(13 \pm 16)
Coke, By-products	81	36.1 \pm 13	9.3 \pm 9	44(17 \pm 16)	20(23 \pm 20)
Coke, Bottom	101	34.5 \pm 12	8.7 \pm 8	35(21 \pm 18)	27(14 \pm 11)
Coke, Top & Side	<u>113</u>	<u>41.2\pm11</u>	<u>13.6\pm9</u>	<u>45(25\pm20)</u>	<u>27(18\pm19)</u>
TOTAL	699				

Those who had worked in more than one department were excluded from analysis. The 236 crane operators worked on overhead cranes in various departments. Generally these 236 men were exposed to atmospheres which were relatively free from dusts, smoke and fumes. The 168 men labelled as masonry workers were involved in masonry work throughout the plant but not on the coke ovens; this group also included foundry workers. Of the workers tested, 295 were involved in the production of coke,

collection of by-product gases, and maintenance of the ovens. They were divided into three groups according to the amount of time they spent in proximity to the most contaminated areas of the oven, the sides, or bench, and the top. The average age of the workers in each group differed somewhat, reflecting the fact that crane operation and jobs on the top and sides of the oven carry higher pay scales and are held by workers with greater seniority. Workers were also classified as current smokers; ex-smokers, if they had stopped more than one month prior to testing; or nonsmokers, if they had never smoked more than 1 to 2 cigarettes per day for any extended period. The fractional distribution of smoking categories was similar within each job category. The average cumulative pack-years of cigarette exposure (1 package per day smoked for 1 year equals a pack-year) roughly reflected the ages of the men.

PULMONARY FUNCTION TESTS

At the time of completion of the questionnaires, men were subjected to a battery of tests. Spirometry was performed using a 13.5-liter water-filled spirometer and standard techniques. Several spirometric indices were calculated including the vital capacity, timed vital capacity or forced expiratory volumes (FEV_{1.0} and 3.0 seconds) and maximum mid-expiratory flow rate (MMFR). The spirometer was equipped with a linear transducer and subjects exhaled into a very low resistance pneumotachygraph. Outputs from these devices were recorded using a rapid X-Y recorder for inscription of a flow-volume loop (Saccomanno et al., 1963). Forced expiratory flow rates were determined at volumes equivalent to 50% (FEF₅₀) and 75% (FEF₇₅) of the vital capacity. Functional residual capacity (FRC) was measured by the helium dilution method. Residual volume (RV) was calculated as the difference between FRC and the expiratory reserve volume determined during spirometry. Total lung capacity (TLC) was measured as the sum of the RV and vital capacity. Other tests obtained, and not further discussed here, included closing volume and oscillatory airway resistance measurements.

Table 2 summarizes selected lung function test results in the 699 workers. Mean values are given for workers grouped according to smoking category. Because of the major influence of age and height on these indices, the mean values shown for each group have been adjusted for group differences in these factors. The statistical significance of differences between group mean values was evaluated by an analysis of variance (Anova).

TABLE 2. PULMONARY FUNCTION TEST RESULTS IN
WORKERS GROUPED BY SMOKING CATEGORY
(AGE AND HEIGHT-ADJUSTED GROUP MEAN VALUES)

	<u>N</u>	<u>VC</u>	<u>FRC</u>	<u>RV</u>	<u>TLC</u>	<u>FEV₁</u>	<u>MMFR</u>	<u>FEF75</u>
Nonsmoker	229	4.94	2.59	1.47	6.47	3.72	231	1.48
Ex-smoker	168	4.95	2.62	1.57	6.60	3.67	217	1.29
Smoker	302	4.75	2.75	1.62	6.43	3.45	201	1.23
P (Anova)		<0.005	<0.05	<0.01	>0.2	<0.001	<0.001	<0.001

As has been repeatedly demonstrated for various populations, smoking status has a significant effect on lung function ($p < 0.01$). Smokers demonstrated lower vital capacity and higher functional residual capacity and residual volume values. Expiratory flow rates were all lower in smokers. The maximum mid-expiratory flow rate and forced flow rate at 75% of vital capacity were respectively 13 and 16% lower in smokers than in nonsmokers. The one second timed vital capacity was also lower, but the difference of only 7% between the mean values in smokers and nonsmokers probably reflects the superiority of the former two indices in detecting small airway changes, the type of abnormality thought to be characteristic of smokers.

Based on the self-completed questionnaires, the workers' respiratory symptoms were graded in severity using a 0 to 7 scale (Mittman et al., 1974). Category 0 included men who had no respiratory complaints. Grades 1 and 2 included men who suffered acute episodes of cough and sputum production but in whom symptoms were present for fewer than 3 months of the year and/or less than 2 years. When respiratory complaints were more persistent or longstanding, a diagnosis of chronic bronchitis was made (grades 5 to 7). The higher grades were reserved for men with chronic cough and sputum production who also suffered from shortness of breath. Grades 3 and 4 were used for symptom complexes of intermediate severity. Table 3 summarizes the pulmonary function test results for workers grouped by the severity of their symptoms. Group mean values were adjusted for any differences in age, height and smoking category. The observed small differences in lung volume measurements were not statistically significant. However, as expected, more severe symptoms were associated with significant

reductions in all flow rate measurements ($p<0.001$). The differences in mean flow rates between asymptomatic workers and those with the most severe symptoms were of the same magnitude as the differences between nonsmokers and smokers.

TABLE 3. PULMONARY FUNCTION TEST RESULTS IN WORKERS
GROUPED BY THE SEVERITY OF RESPIRATORY SYMPTOMS
(AGE, HEIGHT AND SMOKING CATEGORY ADJUSTED GROUP MEAN VALUES)

Symptom Category	<u>N</u>	<u>VC</u>	<u>FRC</u>	<u>RV</u>	<u>TLV</u>	<u>FEV₁</u>	<u>MMFR</u>	<u>FEV₇₅</u>
0	450	4.89	2.66	1.56	6.52	3.64	221	1.35
1, 2	87	4.89	2.75	1.52	6.34	3.68	232	1.50
3, 4	90	4.72	2.60	1.60	6.40	3.43	204	1.26
5, 6, 7	72	4.73	2.68	1.56	6.30	3.35	183	1.06
P (Anova)	>0.1	>0.1	>0.5	>0.1	<0.001	<0.001	<0.001	<0.001

Table 4 summarizes the group mean lung function test results and symptom scores (0 to 7 scale) for workers grouped by work-site. Again, mean values were adjusted for group differences in age, height and smoking status. We anticipated that there would be more frequent and severe respiratory symptoms among coke oven workers and that lung function would be worse in the more exposed men. The former expectation was substantiated; the mean symptom scores were significantly greater in coke workers than in crane operators ($p<0.001$). Mean lung function test results deviated likewise, but the only statistically significant trends involved a fall in vital capacity and the other lung volume indices ($p<0.001$). We did see a tendency to lower flow rate measurements in the more exposed coke workers, but the differences were small and not statistically significant. Thus, the lung function changes observed were those of restrictive lung diseases, such as pulmonary fibrosis, and were not those characteristic of the obstructive changes seen in chronic bronchitis.

TABLE 4. PULMONARY FUNCTION TEST RESULTS AND SYMPTOM SCORES IN WORKERS GROUPED BY WORKSITE
(AGE, HEIGHT AND SMOKING CATEGORY ADJUSTED GROUP MEAN VALUES)

<u>Worksite</u>	<u>N</u>	<u>VC</u>	<u>FRC</u>	<u>RV</u>	<u>TLC</u>	<u>FEV₁</u>	<u>MMFR</u>	<u>FEF₇₅</u>	<u>Symptom Score (0-7)</u>
Crane	236	4.86	2.84	1.73	6.77	3.61	212	1.47	.79
Masonry	168	4.68	2.84	1.75	6.68	3.60	213	1.30	.95
Coke, By-products	81	4.71	2.64	1.42	6.36	3.68	231	1.42	1.01
Coke, Bottom	101	4.63	2.50	1.39	6.25	3.56	211	1.27	1.31
Coke, Top & Side	113	4.55	2.44	1.39	6.15	3.50	217	1.31	1.85
P (Anova)		<0.001	<0.001	<0.001	<0.001	>0.2	>0.2	>0.2	<0.001

To further evaluate these observations the data were analyzed separately for smokers and nonsmokers. Table 5 summarizes the effect of worksite on symptom score; the differences observed were significant for smokers only ($p<0.005$). Similar analyses of the pulmonary function test results failed to show differences between smokers and the nonsmokers or past smokers. Thus, it appears that if smoking plays a synergistic role, it is most apparent in terms of the presence and severity of respiratory symptoms. The small differences in lung function which are seen in the workers most exposed to the coke oven contaminants are present in smokers and nonsmokers alike. It is important to stress that mean values for lung function tests in each worker group were corrected for any differences in smoking category in the groups. Thus, the differences observed, although small, represent the separate or added influence of the toxic effects of the worksite on the more clearly demonstrated smoking effect.

TABLE 5. SYMPTOM SCORES IN WORKERS GROUPED BY
WORKSITE AND CALCULATED SEPARATELY FOR
SMOKERS VERSUS NON- AND EX-SMOKERS

	<u>N</u>	Smokers <u>Symptom Score</u>	<u>N</u>	Non- and Ex-Smokers <u>Symptom Score</u>
Crane	111	1.53	125	0.7
Masonry	69	1.35	99	0.6
Coke, By-products	36	1.63	45	0.9
Coke, Bottom	35	2.31	66	1.0
Coke, Top & Side	51	2.78	62	1.3
P (Anova)		<0.005		>0.1

SPUTUM CYTOLOGY

The development of chronic bronchitis must be associated with structural or histological changes in the lining cells of the tracheobronchial tree. To search for such effects we undertook to perform sputum cytology studies in these workers. At the time of each worker's visit to the laboratory and on completion of the lung function tests, an induced sputum sample was obtained. Workers were exposed for 5 minutes to a mist of hypertonic saline solution generated into a face mask by an ultrasonic nebulizer. Immediately following this procedure, subjects coughed a sputum sample into fixative and these specimens were later prepared for cytological studies (Saccomanno et al., 1963). Slides were read by trained personnel who had no knowledge of the worker's job, smoking status or other characteristics. Various features were tabulated using a standardized recording form. The significance of differences in the occurrence rate of findings in sputum in the various groups was determined by the Chi square test. Table 6 summarizes some of the findings in groups of workers divided by smoking status. Comparing smokers to non or exsmokers, sputum samples in more subjects in the latter two groups were found to contain histiocytes or abnormal giant cell histiocytes or ciliated cells ($p<0.005$). These are the types of desquamated cells expected in the secretions of individuals exposed to irritating agents.

TABLE 6. SPUTUM CYTOLOGY FINDINGS IN WORKERS
GROUPED BY SMOKING CATEGORY

<u>Smoking Category</u>	<u>N</u>	% Workers with Positive Findings			
		<u>Histiocyt es</u>	<u>Abnormal Histiocyt es</u>	<u>Ciliated Cells</u>	<u>Metaplasia</u>
Non-smoker	136	62	12	18	20
Ex-smoker	105	70	22	19	21
Smoker	208	81	34	33	28
P (Chi square)		<0.001	<0.001	<0.005	<0.2

However, to our knowledge, comparable data have never been reported previously. There was a gradient observed in the fraction of workers found to have metaplastic or dysplastic changes in cells in their sputum, but the differences were small and not statistically significant. Differences in the cytology findings in the sputum of workers grouped by symptom scores were similar to the findings shown in Table 6 for the groupings by smoking status. It should be noted that positive findings were relatively common even in nonsmokers, suggesting that irritating factors other than cigarettes were responsible for some of these findings. Table 7 summarizes data related to the possible influence of exposures to the atmosphere at the worksite; the fraction of men in each worksite group found to have large numbers of histiocytes in their sputum did increase as a function of the degree of exposure. A significant gradient ($p<0.05$) was observed for this cytological feature but for no other one. Thus, as with pulmonary function tests, we saw an influence of worksite exposure on cytological findings which was less striking than the differences related to cigarette smoking. It seems unlikely that the worksite environment alone accounted for the high degree of abnormalities found in nonsmokers. Other possible factors, such as ambient air pollution or other exposures at home or in the environment, can also be implicated but cannot be separately evaluated at present.

TABLE 7. SPUTUM CYTOLOGY FINDINGS IN WORKERS
GROUPED BY WORKSITE

<u>Work Area</u>	<u>N</u>	<u>% Workers with 3 or 4+ Histiocytes</u>
Crane	108	14
Masonry	104	21
Coke, By-products	48	29
Coke, Bottom	68	32
Coke, Top & Side	85	30
P (Chi square)		<0.05

CONCLUSIONS

These preliminary data demonstrate an effect of worksite atmospheric exposure(s) on lung structure and function which, although small, is detectable above and beyond the influence of smoking. These observations raise many questions which we cannot answer at this time. For example, the data are insufficient to determine if there is an effect of duration of employment on the test results. Neither could we sort out effects where men were exposed to more than one worksite environment. The data on symptoms suggest that smoking has a synergistic effect in combination with toxic work exposures. The lung function test and cytology results give conflicting indications. These questions, too, cannot be definitely resolved. We have no adequate explanation for the restrictive pattern in lung function tests of some of the more exposed coke oven workers. These data are only suggestive, but perhaps this exposure can produce a restrictive or fibrogenic process, a finding not previously suspected.

We hope that the much more potent study design afforded by longitudinal observations of these workers will resolve some of these issues. Starting from the baseline values established for each worker, annual assessments of changes in structure and function should be more revealing. Perhaps, in combination with the types of industrial hygiene evaluations now being conducted, we will be able to demonstrate dose-response relationships. Those of us conducting human studies have much yet to learn through appropriate application of the lessons taught by classical toxicology studies.

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EPIDEMIOLOGICAL STUDIES OF CANCER
MORTALITY IN COKE PLANT WORKERS

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INTRODUCTION

In 1961 the Department of Biostatistics, University of Pittsburgh, initiated a study of the relationships between job exposures and mortality among the approximately 59,000 men employed at seven Allegheny County steel plants in 1953. During the first phase of the study, follow-up for mortality was carried out through 1961. When the observation of an unusual cancer mortality experience among coke oven workers became apparent, the scope of the investigation was expanded to ten plants throughout other parts of the United States and Canada, while the follow-up on the Allegheny County cohort was extended through 1966. Subsequently, we have updated the employment histories and mortality of the Allegheny County steelworkers through December 31, 1970, and are currently in the process of extending the observation period through 1975.

The major aims of this paper are:

- 1) To summarize the most important findings of these epidemiological studies of mortality among coke oven workers, comparing the results of the different phases of the study.
- 2) To discuss the problems encountered in attempting to evaluate the nature and magnitude of a dose-response relationship between exposure to coke oven effluents and respiratory cancer.
- 3) To present a brief overview of epidemiologic and experimental results linking coal tar exposures to cancers of various anatomic sites.

MATERIALS AND METHODS

The specific details of the design and much of the results for our studies of coke oven workers have been presented in a continuing series in the Journal of Occupational Medicine (Lloyd, 1971; Redmond et al., 1972; Lloyd and Ciocco, 1969; Robinson, 1969; Redmond et al., 1969; Lloyd et al., 1970; Lerer et al., 1974; Redmond et al., 1975; Mazumdar et al., 1975; Rockette and Redmond, 1976). The study data included birthdate, birthplace, race, complete detailed work history at the plant, residence in 1953, and date and cause of death when applicable.

The distribution of the Allegheny County steelworkers by plant and vital status as of December 31, 1970, is shown in Figure 1. Underlying causes for the deaths have been coded by a trained nosologist using the Seventh Revision of the International List (WHO, 1957).

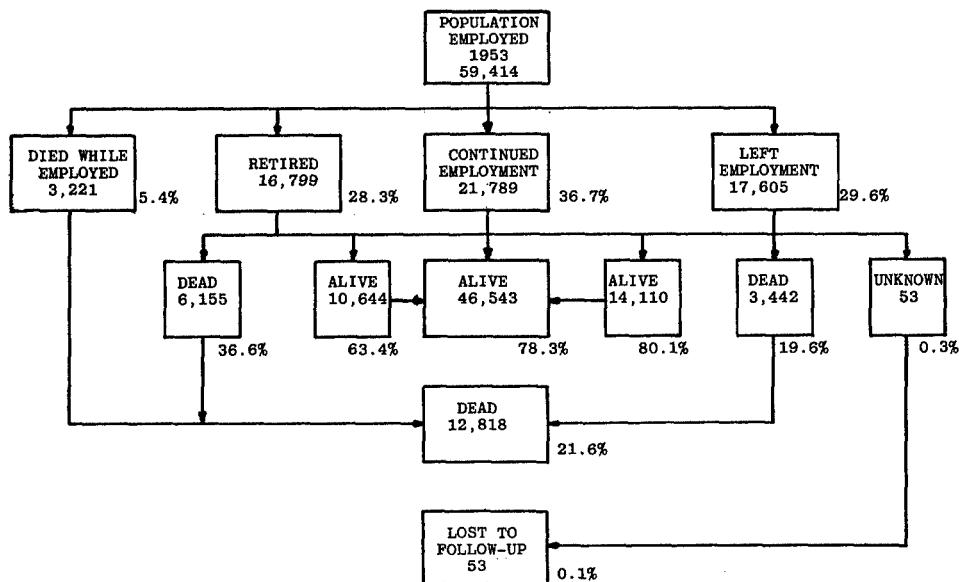


Figure 1. Steelworkers employed in 1953, classified by employment status and vital status through December 31, 1970.

Classification of workers into various categories within the coke plant has been done, with workers in the steel industry who were never in the coke plant being used as the comparison group for calculating expected deaths and mortality ratios. All estimated relative risks have been adjusted for race, age, and calendar period of death. Significance of the relative risks has been assessed by a summary chi-square with one degree of freedom (Mantel, 1966).

BY-PRODUCT COKE PLANT

The primary purpose of the by-product coke plant is the transformation of coal into metallurgical coke, with a secondary function being the recovery of chemical by-products resulting from carbonization. The by-product coke plant consists of three major areas:

- 1) coal-handling
- 2) coke ovens
- 3) by-products plant.

The coke ovens and by-products plant can be further divided by location at the ovens or type of by-product operation. Generally, jobs have been classified into coke oven or nonoven jobs, with the coke oven group including all jobs requiring some or part of the working day spent at the top or side of the coke ovens. For analysis based on duration of exposure a worker may have held jobs in both oven and nonoven areas. Priority has been given to the coke oven experience when assigning a worker to oven or nonoven. Similarly, work at the top of the ovens has been given priority over work at the side of the ovens when considering specific subgroups at the coke ovens. This method of classification clearly delineates the specificity of the lung cancer excess deaths to the jobs at the coke ovens. A certain number of jobs remain unclassified and are designated as "other coke plant" in the tables.

PREVIOUS EPIDEMIOLOGIC STUDIES

It has been known for over 200 years beginning with Percival Pott's (1775) observation of scrotal cancers in London chimney sweeps that some agent produced during combustion of bituminous coal was carcinogenic for the skin of man, while studies beginning in the 1930's demonstrate the carcinogenicity of coal tars for other internal organ systems. An excellent review of the historical studies has been given by Lloyd (Redmond et al., 1969) and will only be summarized briefly here.

Following Pott's observation on chimney sweeps, the next occupational group noted in 1873 to have a risk of scrotal cancer was men involved in the carbonization of lignite (Volkmann, 1875). This was followed by a report of Manouvriez in 1876 that French briquette workers who were exposed to coke oven tar and pitch suffered from scrotal cancer and facial epithelioma (Manouvriez, 1876). Further reports of occupational skin cancers in related occupations followed, leading Great Britain in 1907 to include scrotal epithelioma and epitheliomatous cancer of the skin related to exposure to coal tar compounds under the Workman's Compensation Act.

In 1907 the first report of skin cancers among carbon workers in the United States was published (Lueke, 1907). Animal studies beginning in the early 1900's eventually resulted in the isolation of 3,4 benzpyrene, a potent skin carcinogen (Passey, 1922).

Observations dealing with cancers of other organ sites in association with coal tar or distillate exposures began to appear in the 1930's. Both Japanese (Kuroda and Kawahata, 1936) and British (Kennaway and Kennaway, 1936) producer gas workers were reported to show excesses in lung cancer in 1936. The earliest study of coke oven workers published by Reid and Buck (1956) described a negative finding for lung cancer in retirees, a result which may be partly attributable to study design and partly to our subsequent observation that the extremely high risk is limited to a small proportion of all coke oven workers. An unpublished report in 1960 by Phair and Stirling (1960) dealing with competing causes of death among coal tar workers cites a negative finding for white workers (22 observed deaths versus 22 expected), but a three fold excess for nonwhite workers (17 observed deaths versus 5.8 expected). This rather puzzling excess was found on further subdivision of the workers to be confined to Allegheny County, Pennsylvania, workers in coke production and handling. The lack of consistency in the results, coupled with certain methodological limitations of the study, led the authors to question the reliability of this observation.

COHORT STUDIES OF COKE OVEN WORKERS

In 1971 the first paper dealing with the approximately 3,500 coke plant workers in the Allegheny County steelworkers study appeared (Lloyd, 1971). The major findings reported by Lloyd included:

- 1) An excess mortality risk from lung cancer among coke plant workers, which was confined to men employed at the coke ovens. The greatest risk occurred among topside workers, where the estimated relative risk was ten-fold among men with five or

more years at the top of the ovens. The risk is apparently limited to nonwhite workers, but an examination by length of exposure at the top of the ovens showed that topside oven workers at that time in the Allegheny County plants were primarily black. This observation suggested that lack of sufficient exposure to produce lung cancer might explain the negative finding for whites.

2) An excess risk of certain digestive cancers occurred in nonoven coke plant workers, but the numbers of deaths were too small to attempt to delineate the risk further.

Because of the need to define more fully the lung cancer risk among coke oven workers, particularly as related to racial and geographic differences, the study was expanded in the late 1960's to 10 additional plants. For these plants the study population was limited to all coke oven workers and a sample of other workers in the plants matched by race and starting date of employment to the coke oven workers. In addition, the mortality observation period for the original Allegheny County steelworkers population was extended through 1966.

Examination of the mortality for the 4,661 coke oven workers revealed that (Lloyd et al., 1970):

1) The excess lung cancer risks among white and nonwhite workers are the same when length and area of employment at the ovens are taken into account.

2) The excess risk noted for Allegheny County workers occurs in other geographic areas as well.

3) A finding of a significant excess in kidney cancer deaths became apparent with the larger cohort available for study.

With the updated findings for the Allegheny County steelworkers it was possible to investigate in greater detail the cause of specific cancer mortality among nonoven coke plant workers. Conclusions of note from these analyses are (Redmond et al., 1976):

1) Cancers of the digestive system are significantly elevated in nonoven workers. Cancers of the colon and pancreas account for the entire excess. The excess risk did not appear to cluster in any specific job group included in nonoven.

2) Cancers of the buccal cavity and pharynx appeared high in nonoven workers, although the number of deaths was small.

We have since extended the observation period for the Allegheny County steelworkers through 1970. The findings have not yet been published, but analysis of data on the coke plant workers has recently been completed and will be discussed briefly here.

Table 1 gives the distribution of workers in the coke plant by work area and years employed. Earlier publications have generally shown comparisons of the cause specific relative risks for exposures greater than or equal to five years; however, with the longer period of follow-up and the aging of the cohort, the greater number of deaths makes it possible to consider finer length of exposure breakdowns. Hence, the distribution of workers with ten or more and fifteen or more years is also shown. Note that the number of workers with fifteen or more years experience at the top of the ovens is only 29.

TABLE 1. DISTRIBUTION OF ALLEGHENY COUNTY COKE PLANT WORKERS IN 1953 BY WORK AREA AND LENGTH OF EMPLOYMENT THROUGH 1953

<u>WORK AREA</u>	<u>Years Employed Through 1953</u>		
	<u>5+</u>	<u>10+</u>	<u>15+</u>
Total Coke Plant	1860	1194	790
Coke Oven	993	574	325
Oven Topside Full-Time	150	72	29
Oven Topside Part-Time	290	245	159
Oven Side Only	553	257	137
Nonoven	836	578	392
No One Coke Plant Area	31	42	73

Examination of the cause specific mortality, 1953-1970, reveals a statistically significant 11% excess in all causes among coke plant workers with five or more years experience. As expected, this increase is attributable primarily to the 47% excess cancer mortality. In addition, however, a slight increase in other nonmalignant respiratory diseases has now become statistically significant. The estimated excess in this

category is also 47%. Table 2 provides a summary of the cancer mortality risks by location and duration of exposure in the coke plant. The level of excess risk attained during the various updates of the study has remained relatively stable with the lung cancer mortality elevated for workers in all subdivisions of the coke ovens. There is a consistent increase in the level of risk with increased length of exposure for each of the coke oven groups. This same pattern does not hold true for the nonoven workers.

TABLE 2. OBSERVED DEATHS AND RELATIVE RISKS OF DEATH FROM MALIGNANT NEOPLASMS, 1953-1970, FOR COKE PLANT WORKERS BY WORK AREA AND LENGTH OF EMPLOYMENT THROUGH 1953

Work Area	Years Employed Through 1953					
	5+		10+		15+	
	Obs.	Rel. Risk	Obs.	Rel. Risk	Obs.	Rel. Risk
Total Coke Plant	166	1.47**	136	1.50**	108	1.62**
Coke Oven	101	1.66**	85	1.95**	63	2.40**
Oven Topside Full-Time	35	3.70**	22	5.12**	12	7.63**
Oven Topside Part-Time	26	1.59*	31	1.85**	32	2.73**
Oven Side Only	40	1.17	32	1.46	19	1.51
Nonoven	65	1.28	48	1.10	39	1.13
No One Coke Plant Area	0	--	3	--	6	1.34

* p < .05

** p < .01

-- Less than 5 deaths.

Table 3 shows the relative risks for lung cancer. Here, a strong relationship is observed for increased risks related to longer durations of exposure. Among the 29 workers with fifteen or more years topside experience, 8 (28%) died of lung cancer. For side oven workers, whereas the risk was not statistically significant during the earlier phases of the study, the risk of 1.79 is significant with the addition of the 1967-1970 mortality.

TABLE 3. OBSERVED DEATHS AND RELATIVE RISKS OF DEATH FROM CANCERS OF THE RESPIRATORY SYSTEM, 1953-1970, FOR COKE OVEN WORKERS BY WORK AREA AND LENGTH OF EMPLOYMENT THROUGH 1953

<u>Work Area</u>	<u>Years Employed Through 1953</u>					
	<u>5+</u>		<u>10+</u>		<u>15+</u>	
	<u>Obs.</u>	<u>Rel.</u> <u>Risk</u>	<u>Obs.</u>	<u>Rel.</u> <u>Risk</u>	<u>Obs.</u>	<u>Rel.</u> <u>Risk</u>
Coke Oven	54	3.02**	44	3.42**	33	4.14**
Oven Topsid Full-Time	25	9.19**	16	11.79**	8	15.72**
Oven Topsid Part-Time	12	2.29**	16	3.07**	18	4.72**
Oven Side Only	17	1.79*	12	1.99**	7	2.00

* p < .05

** p < .01

The relative risks of dying from cancers of the digestive organs among nonoven workers are presented in Table 4. This table indicates that the excesses previously noted for cancers of the large intestine and pancreas have not increased during the most recent follow-up. For the colon cancers there appears to be no increase in risk with increasing exposure. The apparent absence of a dose response relationship, albeit based on small numbers, makes the interpretation of the findings more difficult.

TABLE 4. OBSERVED DEATHS AND RELATIVE RISKS OF DEATH FROM CANCERS OF THE DIGESTIVE SYSTEM, 1953-1970, AMONG NONOVEN WORKERS BY LENGTH OF EMPLOYMENT THROUGH 1953

<u>Cause of Death</u>	<u>Length of Employment</u>					
	<u>5+</u>		<u>10+</u>		<u>15+</u>	
	<u>Obs.</u>	<u>Rel.</u> <u>Risk</u>	<u>Obs.</u>	<u>Rel.</u> <u>Risk</u>	<u>Obs.</u>	<u>Rel.</u> <u>Risk</u>
All Malignant Neoplasms of Digestive System	28	1.58*	23	1.53	19	1.53
Large Intestine	11	2.31*	10	2.52**	8	2.37*
Pancreas	8	3.67**	7	3.75**	6	4.29**
Other	9	0.83	6	0.65	5	0.65

* p < .05

** p < .01

Table 5 provides a summary of the observed deaths and relative risks of dying from other respiratory diseases. Contrary to the findings for respiratory cancer mortality, the risks for oven and nonoven workers are about the same. The magnitude of the risks increases markedly among workers with longer durations of exposure suggesting a direct relationship to the occupational environment. However, the lack of specificity to any particular work area or occupational group complicates the exposure pattern.

TABLE 5. OBSERVED DEATHS AND RELATIVE RISKS OF DEATH FROM NONMALIGNANT RESPIRATORY DISEASES, 1953-1970, FOR COKE PLANT WORKERS BY WORK AREA AND LENGTH OF EMPLOYMENT THROUGH 1953

Work Area	Years Employed Through 1953					
	5+		10+		15+	
	Obs.	Rel. Risk	Obs.	Rel. Risk	Obs.	Rel. Risk
Total Coke Plant	34	1.47*	31	1.82**	25	2.01**
Coke Oven	20	1.47	17	1.92*	12	2.20*
Nonoven	14	1.45	14	1.75	13	2.07*
No One Coke Plant Area	0	--	0	--	0	--

* p < .05

** p < .01

-- Less than 5 deaths.

DISCUSSION

In attempting to assess causal relationships from observational data, the epidemiologist must rely on certain criteria to assist in separating causation from mere association of events of concern. These criteria include the consistency of the association using a variety of methods of approach, the strength and specificity of the association, the appropriate temporal relationship of cause to effect, and the coherence of the epidemiological evidence with experimental and other biological knowledge. Evaluated according to these criteria the cancer patterns among the coke oven workers present convincing evidence of a cause effect relationship.

A few points are worthy of mention here which may provide additional insight or suggest avenues for further inquiry. Lloyd (Redmond et al., 1969) summarizes some data on mortality from scrotal cancer (Table 6) which indicates that the carcinogenic compound(s) may become more concentrated with successive distillations. He also points out that the lung cancer mortality excess appears to be positively associated with the temperatures involved in the carbonization process (Table 7).

TABLE 6. MINIMAL ESTIMATES OF MORTALITY RATES FROM CANCER OF THE SCROTUM FOR OCCUPATIONS WITH EXPOSURE TO COAL TAR PRODUCTS*

	Mortality Rate (x 10 ⁶)
Chimney sweeps	754.7
Patent fuel workers	504.2
Tar distillery workers (excluding those at gas works)	212.9
Skilled makers of coal gas and coke	123.9
All coke oven workers	21.1
Producer gas men	10.9
Dyers and dyers laborers	7.9
General population	4.2

*Henry, S. A., Cancer of the Scrotum in Relation to Occupation, London, Oxford University Press, 1946.

TABLE 7. TEMPERATURE RANGE OF CARBONIZING CHAMBERS
AND EXCESS OF LUNG CANCER REPORTED

<u>Carbonizing Chamber</u>	<u>Temperature Range</u>	<u>Percent Excess of Lung Cancer Reported</u>
Vertical retorts	400 - 500 C	27%
Horizontal retorts	900 -1100 C	83%
Coke ovens	1200 -1400 C	255%
Japanese gas generators	\geq -1500 C	800%

Another question which frequently arises in any observed association of lung cancer to exposure is the role of cigarette smoking. Unfortunately, as is true with most long-term follow-up studies, no smoking histories are available. However, it is possible to consider whether the observed differences in lung cancer mortality among coke oven workers might be explained by smoking habits. Table 8 contains age specific lung cancer mortality rates for U.S. males by smoking habits in comparison to age specific rates for coke oven workers and all steelworkers (Lloyd, 1974). It appears that the steelworkers population as a whole has rates which resemble heavy smokers, while the lung cancer rates for topside coke oven workers are considerably greater than what could be due to differential smoking habits. While we cannot rule out synergism of these two variables, nonetheless cigarette smoking alone cannot explain the lung cancer experience of coke oven workers.

TABLE 8. ESTIMATES OF AVERAGE ANNUAL LUNG CANCER MORTALITY RATES (PER 100,000 PERSON-YEARS) FOR SELECTED U.S. SMOKING GROUPS, 1954-1962 AND STEELWORKER GROUPS, 1953-1961

	U. S. Smokers	35-44	45-54	55-64	65-74
		<45	45-54	>55	
Never smoked or occasional only		-	-	12	29
Current cigarette smokers, total	5	39	158	258	
Current cigarette smokers, 1-9/day	-	-	69	119	
Current cigarette smokers, over 39/day	-	104	321	559	
Steelworkers		12	126	160	
Coke oven, never topside	-	130	387		
Coke oven, topside	228	1,058	1,307		
Coke oven, \geq 5 years topside	265	1,587	1,961		

Efforts to develop more specifically a dose response curve of the relationship of lung cancer to coke oven effluents are in progress. One paper describing the analysis of coal tar pitch volatiles (CTPV-mg/m³) as related to lung cancer using a cumulative time weighted index has been published (Mazumdar et al., 1975). In further work we have incorporated the concept of a latent period using a log normal distribution for the latent times. Other simple non-threshold dose response models have been considered, with emphasis on the response at low dose values. Always extrapolation is involved as no workers were exposed at doses as low as those of interest for standard setting purposes. Although these analyses are not yet complete, the general results would seem to indicate that for average levels of CTPV around 0.2 mg/m³ of air, the risks may not be negligible, even for the dose response models incorporating latent periods which give the lowest estimates of risk. From the standpoint of setting standards, clear difficulties exist if the standard is to be based solely on available epidemiological data.

In spite of considerable experimental data concerning the carcinogenicity of benzpyrene and other compounds within the coke oven effluents, the specific carcinogens or mixtures responsible for the excess cancers in the coke plant workers is unclear. The picture is made more difficult to clarify due to the possible involvement of multiple organ sites, e.g. skin, lung, kidney, pancreas, and colon.

SUMMARY

This paper describes the results of recent epidemiological studies of mortality in coke plant workers. A general consistency in the findings among various studies is noted with evidence for a cause effect relationship discussed. The studies of coke plant workers provide information indicating cancers of multiple organ sites occur subsequent to exposures to coal tar and its distillates.

Work on developing an appropriate dose response relationship is discussed briefly; the models indicate that even low doses may result in increased lung cancer risk.

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MUTAGENIC EVALUATION OF 1,1-DIMETHYLHYDRAZINE,
METHYLHYDRAZINE AND N-PHENYL- α -NAPHTHYLAMINE

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INTRODUCTION

N-phenyl- α -naphthylamine and monomethyl- and dimethyl-derivatives of hydrazine are entering our environment as components of jet and rocket fuels and therefore have been identified as a potential risk to the environment in general and to workers handling these products (Epstein and Shafner, 1968; Greenhouse, 1967).

Hydrazines and methylhydrazine have been shown to react with pyrimidine bases, especially at a high pH, to break the pyrimidine ring and cause removal of the base from DNA (Freese, 1971). Based on this type of reaction with DNA, the potential for hydrazine and/or its derivatives to exhibit mutagenic, teratogenic and carcinogenic activity might be expected.

Some evidence for this concern exists. Hydrazine was mutagenic in phage (Freese et al., 1961), Salmonella typhimurium (Ames, 1971), Drosophila melanogaster (Shukla, 1972; Jain and Shukla, 1972), Lycopersicum (Jain et al., 1968), and in the host-mediated assay using S. typhimurium strain G-46, but was not active in the dominant lethal assay (Epstein and Shafner, 1968; Röhrbörn et al., 1972). Unsymmetrical dimethylhydrazine also induced mutation in S. typhimurium TA-1535 but was negative in the micronucleus test and sperm head abnormality tests (John Heddle, personal communication). Symmetrical dimethylhydrazine induced mitotic gene conversion in yeast (Zimmerman and Schwaien, 1967) but did not induce reverse point mutations in S. typhimurium (Ames, personal communication). Hydrazine, its monomethyl derivatives, and N-phenyl- α -naphthylamine have also been shown to be teratogenic in embryos of the South African Clawed Toad, Xenopus laevis (Greenhouse, 1967).

In addition, hydrazine in relatively high doses has been shown to cause leukemia, reticulum cell sarcomas, lung adenomas (Juhasz, 1967) and hepatomas (Biancifioni et al., 1964) in mice.

Because of this accumulating evidence, and because of the strengthening correlation between mutagenicity and carcinogenicity, it seemed prudent to test these compounds in a mutagenicity assay, which in turn might provide insight into potential toxicologic problems associated with the test agent.

To test these compounds, we have organized a matrix of assays (Figure 1) employing microbial assays, mammalian cells in culture, and dominant lethality in rats and mice. This battery of tests can detect forward and reverse point mutations, chromosomal aberrations, and mitotic recombinational events induced by acute and subchronic exposure to the test substances. This testing program is not only sensitive and reproducible, but also includes in vivo tests relevant to normal exposure and pharmacologic conditions encountered in the environment. We should also advance the additional argument that if a single toxicological endpoint such as mutation can be demonstrated among several different test species, then application of the response to a wider range of species, including man, can be made.

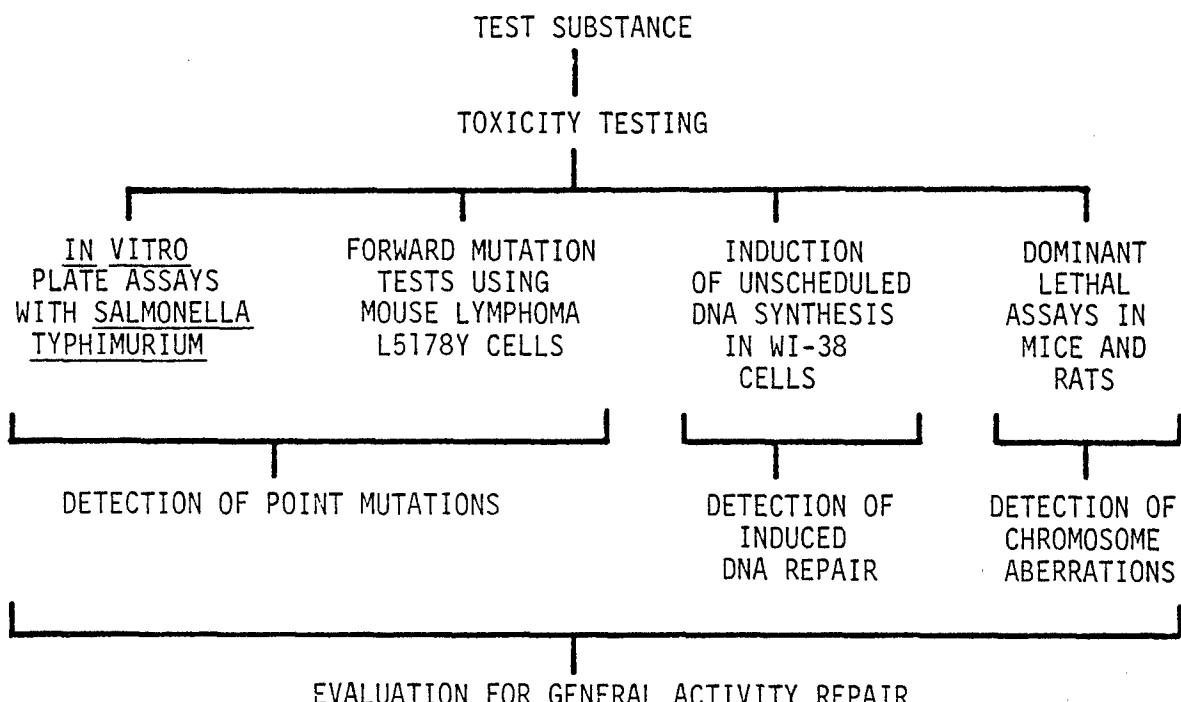


Figure 1. Composition of the genetic evaluation program.

MATERIALS AND METHODS

In Vitro Microbial Assays

The test chemicals were examined in a series of microbial assays employing histidine-requiring mutants of S. typhimurium. The assays were conducted so that the compound was tested directly and in the presence of a mouse liver microsome activation system.

The compounds were evaluated at a minimum of four dose levels under both test conditions with the highest dose level showing some evidence of toxicity. In addition to these tests, spot tests (Ames, 1971) were conducted with the Salmonella mutants plus additional strains of bacteria; S. typhimurium strain G-46 and E. coli strain WP₂uvrA⁻ (Greene and Muriel, 1976).

Overnight cultures of S. typhimurium (G-46, TA-1535, TA-1537, TA-1538, TA-98 and TA-100), E. coli (WP₂uvrA⁻) and S. cerevisiae (D4) were grown in complete broth. All cultures were monitored regularly for stability of markers and for contamination. Approximately 10^8 cells from a culture were added to test tubes containing 2.0 ml of molten agar supplemented with biotin and a trace amount of histidine.

Four dose levels of the test chemical, dissolved in DMSO (dimethylsulfoxide), were added to the appropriate tubes and the contents poured over selective medium. In activation tests 0.5 ml aliquots of the reaction mixture containing the microsomes were added to the tubes containing the cells and chemical just prior to pouring onto the selective medium. After the overlays solidified, the plates were placed in a 37 C incubator for 48 to 72 hours. The plates were then scored for the number of colonies growing in the agar overlay.

Positive control compounds were included as reference points to ensure that the assay was functioning with known mutagens. Direct acting mutagens were employed in nonactivation assays; mutagens requiring microsomal activation were used in activation assays. Supplementary spot tests were also conducted according to the methods described by Ames et al. (1975).

To provide the microbial fractions for the activation system, male mice (sufficient to provide the necessary quantities of tissues) were killed by cranial blow, decapitated and bled. Organs were immediately dissected from the animal using aseptic techniques

and placed in ice-cold 0.25 M sucrose buffered with Tris buffer at a pH of 7.4. Upon collection of the desired quantity of organs, they were washed twice with fresh buffered sucrose and completely homogenized with a motor-drive homogenizing unit at 4 C. The whole organ homogenate obtained from this step was centrifuged for 20 minutes at 9,000 $\times g$ in a refrigerated centrifuge. The supernatant from the centrifuged sample was retained and frozen at -80 C. Samples from these preparations were combined with the following reactive mixture and used for the activation studies.

<u>Component</u>	<u>Final Concentration/ml</u>
TPN (sodium salt)	6 μM
Isocitric acid	35 μM
Tris buffer, pH 7.4	28 μM
MgCl ₂	2 μM
Homogenate fraction equivalent to 25 mg of wet tissue	

In Vitro Mutation Assay in Mammalian Cells

The test chemicals were also evaluated for mutagenic activity in a forward mutation assay employing cultured mouse cells (L5178Y). This cell line is heterozygous for the thymidine kinase (TK+/-) gene, and the assay detects forward mutation to the TK-/- genotype by measuring its ability to form colonies in the presence of BUdR. Compounds were tested directly and in the presence of a mouse liver, post-mitochondrial fraction.

The procedure used was a modification of that reported by Clive and Spector (1975). Briefly, rapidly growing cells were cleansed of spontaneous Tk-/- mutants by growing them in a medium containing thymidine hypoxanthine, methotrexate and glycine (THMG). Only cells which produce the enzyme thymidine kinase can utilize the exogenous thymidine from the medium and grow.

The test compounds were dissolved in DMSO and diluted in growth medium (F_{10-p}) consisting of Fischer's Medium for the Leukemic Cells of Mice supplemented with horse serum and sodium pyruvate. Ten doses of the test substances were selected and added to tubes containing 3×10^6 cleansed cells in F_{10-p}. DMSO was used as the solvent control and ethylmethane sulfonate was included in the series as a known direct acting mutagen.

An activation series combining 2.5 ml of the reaction mixture described for the microbial series with the cells and test substances in 10 ml of F10-p was run simultaneously with the nonactivation series. Dimethylnitrosamine, a strong mutagen requiring activation, was used as the positive control compound.

After five hours, the cells were twice washed to remove the test substances, fed and allowed to express for three days. During this period, toxicity was monitored from daily cell counts as a loss in growth potential. At the end of the expression period, four doses were selected for cloning from the range of concentrations by using the highest dose that showed no loss in growth potential as the penultimate dose and by bracketing this level with one higher and two lower dose levels.

One million cells from each of these dose levels was plated in a selection medium containing soft agar and the thymidine analogue BUdR (5'xbromodeoxyuridine). This selection medium permitted only cells which had mutated to the TK-/- genotype to survive and form colonies. One hundred cells from each dose level was similarly plated in each of three petri dishes in the soft agar medium without BUdR. These cultures were used to measure cell survival. A mutation frequency could be obtained from the information obtained from both sets of plates.

Unscheduled DNA Synthesis (UDS)

Normal human diploid WI-38 cells obtained from Flow Laboratories were seeded at 5×10^5 cells in a 100 mm tissue culture dish and grown to confluence in Eagle's minimal essential medium (EMEM) supplemented with 10% fetal calf serum (FCS) (GM). Once reaching confluence, the cells were switched to EMEM plus 0.5% FCS (SM) for five days. The contact inhibition imposed by confluence and the use of SM held the cells in a nonproliferating state.

On the day of treatment, cells arrested in the G₁ phase of the cycle were placed in selection medium containing 10^{-2} hydroxyurea (HUM). After 30 minutes, this medium was replaced by 5 ml of HUM containing the control or test chemical and 1.0 μ Ci of ³HTdR. Each treatment was at three concentrations. After 90 minutes, exposure to the compound was terminated by washing the cells twice in cold BSS containing an excess of cold thymidine.

Treated plates were frozen at -20 C until processed. After thawing, the cells on the 100 mm plate were covered with 2.5% SDS in 1 x SSC and scraped from the dish with a rubber policeman. The cells were washed and precipitated from the SDS by three changes of 95% ethanol and centrifuged at 10,000 x g. Additional lipid components were removed by extraction in ethanol-ether (3:1) at 70 C. This pellet was washed in 70% ethanol, further incubated at 70 C in 0.3N NaOH, and the DNA finally extracted in 50 μ l 1N PCA at 70 C. The DNA was separated into two 25 μ l aliquots. One of these was dissolved in 10 ml of Hydromix scintillation cocktail (Yorktown Co.) and counted in a Beckman liquid scintillation spectrometer. The second aliquot was added to 275 μ l of 1N PCA and read at 260 nm in a Gilford spectrophotometer. The values were corrected for light scatter and converted to μ g of DNA. Following liquid scintillation counting, the data were combined with the DNA extraction values and expressed as disintegration per minute per μ g DNA (DPM/ μ g DNA).

The activation tests were conducted according to the methods described above except that 0.62 ml of a purified microsome preparation (105,000 x g pellet) in the reaction mixture described for the microbial assay was added to the test mixture.

Doses were determined from preliminary toxicity tests in which cells were seeded in 16 mm wells (Linbro plate). A wide range of concentrations was tested in the wells, and toxicity was monitored visually by altered cell morphology and loss of cell adhesion. The concentrations of test and control compounds are given in the data tables.

Dominant Lethal Assay (DLA)

The dominant lethal assay is designed to assess the ability of the test compounds or their metabolic products to reach the testes of treated male animals and induce genetic activity in the developing gametes during spermatogenesis.

N-phenyl- α -naphthylamine and 1,1-dimethylhydrazine were administered to male mice, and methylhydrazine was administered to male mice and rats according to the following protocol.

The assay consisted of dosing 10 male mice or rats with the test compound at three dosage levels and with positive and negative control substances for five consecutive days. After a two-day rest, the treated males were each mated with two virgin females. After five days, the females were transferred to new cages. Two days later, two new virgin females were caged with the males for five days. This sequence of repetitive mating to two new females each week was continued for seven weeks.

Fourteen days after the mid-week in which they were caged with the males, the females were killed and dissected. The number of dead and living embryos in the uterus was recorded on standard forms. In rats corpora lutea were also counted and recorded. These data were statistically analyzed for indications of dominant lethality and compared with control data for significance.

All animals were offered a 4½% fat diet and water ad libitum. Water was acidified according to approved laboratory animal health standards.

Male mice were housed five to a cage while being dosed with the compound and then housed separately with two females for mating. Sanitary cages and bedding were used and changed twice a week at which times water containers were cleaned, sanitized and filled. Cages were repositioned on racks once a week, and the racks repositioned within rooms monthly. Personnel handling animals wore suitable garments. Individuals with respiratory or other overt infections were excluded from the animal facility.

The test samples were obtained from the United States Air Force. MH and UDMH were supplied as clear liquids (less than 100 ml) in amber bottles. N-phenyl- α -naphthylamine was supplied as pea-sized pellets.

All compounds were prepared daily from stock. 1,1-dimethylhydrazine and methylhydrazine were dissolved in triple distilled water and diluted. N-phenyl- α -naphthylamine pellets were ground with a mortar and pestle, weighed and dissolved in corn oil at room temperature.

Dosages were determined from LD₅₀ data supplied by the United States Air Force. A high dose of 1/10 the LD₅₀, an intermediate dose at 1/3 the high level, and a low dose of 1/19 the high level were used. Compounds were injected intraperitoneally (i.p.) into each animal daily for five days.

Calculated dosages are as follows:

1,1-dimethylhydrazine

<u>Mice</u>	<u>mg/kg</u>
LD ₅₀	125
High 1/10 LD ₅₀	12.5
Int. 1/30 LD ₅₀	4.3
Low 1/100 LD ₅₀	1.25
	Wt. 30 ± 2.5 gm

<u>Mice</u>	<u>Methylhydrazine</u>
LD ₅₀	26.0
High 1/10 LD ₅₀	2.6
Int. 1/30 LD ₅₀	0.86
Low 1/100 LD ₅₀	0.26
	Wt. 30 ± 2.5 gm

Rats

LD ₅₀	21.5
High 1/10 LD ₅₀	2.15
Int. 1/30 LD ₅₀	0.72
Low 1/100 LD ₅₀	0.215

Wt. 325 ± 25 gm

N-phenyl-α-naphthylamine

Mice

LD ₅₀	1231
High 1/10 LD ₅₀	500*
Int. 1/30 LD ₅₀	156
Low 1/100 LD ₅₀	50

Wt. 30 ± 2.5 gm

*This dose was selected as the high dose by the contract monitor.

Triethylenemelame (TEM) was administered i.p. at a level of 0.3 mg/kg in 0.85% saline as a positive control. Negative control animals received an i.p. injection of the corn oil or water solvents.

RESULTS AND INTERPRETATIONS

1,1-dimethylhydrazine (UDMH), methylhydrazine (MH) and N-phenyl-α-naphthylamine (PANA) were evaluated for genetic activity in a series of assays employing microbial cells, mammalian cells in culture and in vivo studies in rats and mice.

Microbial Assays

These assays are designed to evaluate the test substances for mutagenic and recombinogenic activity in strains of Salmonella and Saccharomyces. They measure the direct action of the chemicals and also their metabolites after conversion by liver metabolic activation systems in semiquantitative plate tests. The results for these assays are shown in Tables 1 and 2.

TABLE 1. RESULTS FROM MICROBIAL ASSAYS EVALUATING THE GENETIC ACTIVITY OF UDMH

Concentration (μ l/plate)	Revertants Per Plate with Indicator Strain						
	TA-1535	TA-1537	TA-1538	TA-98	TA-100	D4	WP ₂ uvrA ⁻
<u>Nonactivation</u>							
Solvent Control	25(-)	16(-)	23(-)	39(-)	146(-)	30(-)	{-}
Positive Control	>10 ³ (+)	>10 ³ (+)	>10 ³ (+)	>10 ³ (+)	>10 ³ (+)	174(+)	{+}
UDMH							
0.01	25	9	27	42	158	39	-
0.1	20	11	25	49	152	32	-
1.0	20	23	29	40	146	31	-
5.0	0(-)	28(-)	17(-)	22(-)	168(-)	27(-)	{-}
<u>Activation</u>							
Solvent Control	26(-)	11(-)	30(-)	42(-)	172(-)	33(-)	{-}
Positive Control	264(+)	240(+)	>10 ³ (+)	>10 ³ (+)	>10 ³ (+)	-	{+}
UDMH							
0.01	20	11	38	39	186	32	-
0.1	21	10	31	52	175	34	-
1.0	31	10	41	74	189	31	-
5.0	10(-)	22(-)	51(-)	100(-)	199(-)	32(-)	{-}

() = Results of qualitative spot test (+) = positive response
(-) = negative response

TABLE 2. RESULTS FROM MICROBIAL ASSAYS EVALUATING THE GENETIC ACTIVITY OF MH

Concentration (μ l/plate)	Revertants Per Plate with Indicator Strain						
	TA-1535	TA-1537	TA-1538	TA-98	TA-100	D4	WP ₂ uvrA ⁻
<u>Nonactivation</u>							
Solvent Control	23(-)	18(-)	21(-)	17(-)	117(-)	31(-)	{-}
Positive Control	>10 ³ (+)	>10 ³ (+)	>10 ³ (+)	440(+)	>10 ³ (+)	172(+)	{+}
MH							
0.0001	16	13	15	25	68	-	-
0.001	27	13	17	21	143	31	-
0.01	20	23	15	22	143	33	-
0.1	16	28	12	12	128	39	-
1.0	0	0	0	0	92	61	{-}
5.0	-(-)	-(-)	-(-)	-(-)	-(-)	-(-)	{-}
<u>Activation</u>							
Solvent Control	32(-)	21(-)	30(-)	72(-)	89(-)	33(-)	{-}
Positive Control	>10 ³ (+)	240(+)	>10 ³ (+)	>10 ³ (+)	>10 ³ (+)	-	{+}
MH							
0.01	31	38	29	48	64	36	-
0.1	33	25	28	51	68	32	-
1.0	41	52	19	56	44	30	-
5.0	31(-)	44	19(-)	65(-)	93(-)	34(-)	{-}

() = Results of qualitative spot test (+) = positive response
(-) = negative response

There were no clear indications of mutagenic activity by UDMH or by MH, although concentrations which consistently produced clear toxicity were used.

Marginal responses for UDMH were noted in the activation tests with Salmonella TA-98 and possibly TA-1538 but none of these responses were sufficiently strong to be considered significant by themselves.

Because hydrazines have certain properties similar to nitrosamines, MH was also examined for mutagenic activity in a suspension assay. Dimethylnitrosamine was found to be inactive in the standard plate assay, but highly mutagenic when tested in a suspension assay. Table 4 shows that mutagenic activity was observed with MH concentrations of 1 μ l and 5 μ l/ml after a 60 minute incubation with a mouse liver activation system.

PANA was also evaluated in the standard plate tests but did not show mutagenic activity (Table 3). β -naphthalamine was tested using the same procedures and was positive in the strain TA-1535. Therefore, we can conclude that PANA does not have β -naphthylamine-like mutagenic properties.

TABLE 3. RESULTS FROM MICROBIAL ASSAYS EVALUATING THE GENETIC ACTIVITY OF PANA

Concentration (μ l/plate)	Revertants Per Plate with Indicator Strain						
	TA-1535	TA-1537	TA-1538	TA-98	TA-100	D4	WP ₂ uvrA ⁻
<u>Nonactivation</u>							
Solvent Control	23(-)		18(-)		21(-)		17(-)
Positive Control	>10 ³ (+)		>10 ³ (+)		325(+)		440(+)
PANA							
0.5	25		18		20		12
5.0	20	1	13		15		13
50.0	12		11		18		12
250.0	22		30		21		10
500.0	-(-)		11(-)		-(-)		-(-)
<u>Activation</u>							
Solvent Control	32(-)		21(-)		30(-)		72(-)
Positive Control	210(+)		240(+)		>10 ³ (+)		>10 ³ (+)
PANA							
0.5	33		28		27		44
5.0	35		30		30		41
50.0	23		35		12		38
250.0	37		37		30		44
500.0	-(-)		27(-)		-(-)		-(-)

() = Results of qualitative spot test (+) = positive response
(-) = negative response

TABLE 4. RESULTS FROM SUSPENSION TESTS OF MH USING
S. TYPHIMURIUM STRAIN TA-1535^a

Test	Compound Concentration/ml	Population Counts	Mutant Counts	Mutation Freq. ($\times 10^{-8}$)
<u>Activation</u>				
Solvent Control (a)	-	2698*	227	8.4
(b)	-	1369**	86	6.3
Positive Control	DMN 100 μ moles	1264	4798	379.6
MH	1 μ l	1043**	332	31.8
MH	5 μ l	1958*	9855	503.3

*

**Identifies treated group with appropriate solvent control.

^aThe suspension assay was conducted using the same mouse liver activation system described for the plate assays shown in Table 1. The protocol was changed such that rather than add all test components to semisolid overlay agar, they were suspended in saline and incubated 60 minutes at 37°C on a rotary shaker. After incubation samples were removed and assayed for the numbers of surviving cells and numbers of revertants. Mutation frequencies were calculated for each test. Dimethylnitrosamine (DMN) was used as the positive control compound.

Mouse Lymphoma Assay

By testing these compounds in a mouse lymphoma assay using the L5178Y strain of cells, further sensitivity was applied to examine the test substances' ability to induce point mutations. The specific event detected by these cells was forward mutation at the thymidine kinase (TK $+$ / $-$ \rightarrow TK $-$ / $-$) locus. The entire gene serves as target for this autosomal recessive trait, and hence should be expected to be more sensitive. Both activation and nonactivation assays were conducted.

The results for UDMH tested in this assay are shown in Table 5. A moderately strong, dose-related response was demonstrated, especially, in the microsome activation tests. In these tests, there was a fifteen (15) fold increase in mutation frequency at 0.1 μ l/ml. Concurrently, the absolute increase in numbers of TK $-$ / $-$ mutants was between two and three times greater than the solvent controls; thus, the selection of preexisting mutants can be ruled out.

TABLE 5. RESULTS OF THE MOUSE LYMPHOMA MUTAGENICITY ASSAY FOR UDMH

Test	A Day 1	B 2	C 3	D 4	E ΔGS	F % GS	G MC	H VC	I % CE	J GF	K MF(10^{-4})
<u>Nonactivation</u>											
Solvent Control	1.5	-	11.1	-	9.6	100	89	191	100	100	0.5
Positive Control	1.6	-	2.3	-	0.7	7	288	7	4	0.3	41.1
UDMH											
0.01 μ l/ml	2.5	-	23.9	-	21.4	223	70	119	62	139	0.6
0.05 μ l/ml	1.6	-	14.0	-	12.4	129	105	98	51	66	1.1
0.1 μ l/ml	2.0	-	20.0	-	18.0	188	102	60	31	59	1.7
0.25 μ l/ml	1.3	-	12.0	-	10.7	111	73	48	25	28	1.5
<u>Activation</u>											
Solvent Control	1.1	-	7.2	-	6.1	100	58	229	100	100	0.3
Positive Control	1.5	-	2.7	-	0.2	3	184	8	3	0.9	2.3
UDMH											
0.005 μ l/ml	1.3	-	28.4	-	27.1	444	63	69	30	134	0.9
0.01 μ l/ml	2.1	-	10.8	-	8.7	143	91	64	28	40	1.4
0.05 μ l/ml	1.0	-	5.5	-	4.5	73	186	89	39	28	2.1
0.1 μ l/ml	2.4	-	5.9	-	3.5	57	146	31	14	8	4.7

The results for MH and PANA, shown in Tables 6 and 7, did not demonstrate an ability of these compounds to induce genetic activity in mouse lymphoma cells.

TABLE 6. RESULTS OF THE MOUSE LYMPHOMA MUTAGENICITY ASSAY FOR MH

Test	A Day 1	B 2	C 3	D 4	E ΔGS	F % GS	G MC	H VC	I % CE	J GF	K MF(10^{-4})
<u>Nonactivation</u>											
Solvent Control	0.1	-	1.1	2.9	2.8	100	137	112	100	100	1.2
Positive Control	1.6	-	2.3	-	0.7	7	288	7	4	0.3	41.1
MH											
0.0005 μ l/ml	1.5	-	3.2	5.8	4.3	153	58	48	43	65	1.2
0.001 μ l/ml	1.3	-	1.6	6.7	5.4	192	120	63	56	107	1.9
0.05 μ l/ml	1.8	-	2.7	6.1	4.3	154	87	60	54	82	1.5
0.1 μ l/ml	1.8	-	2.2	3.6	1.8	64	102	92	82	58	1.1
<u>Activation</u>											
Solvent Control	4.7	-	10.6	-	5.9	100	22	81	100	100	0.3
Positive Control	1.5	-	0.2	-	0.2	3	184	8	3	0.9	2.3
MH											
0.001 μ l/ml	2.9	-	15.8	-	13.9	235	7	65	80	188	0.1
0.005 μ l/ml	4.2	-	13.0	-	9.8	166	11	42	52	86	0.3
0.01 μ l/ml	4.3	-	7.4	-	3.1	53	5	16	20	10	0.3
0.05 μ l/ml	3.0	-	3.7	-	0.7	12	6	12	15	2	0.5

TABLE 7. RESULTS OF THE MOUSE LYMPHOMA MUTAGENICITY ASSAY FOR PANA

Test	A Day 1	B 2	C 3	D 4	E ΔGS	F % GS	G MC	H VC	I % CE	J GF	K MF(10^{-4})
<u>Nonactivation</u>											
Solvent Control	1.5	-	11.1	-	9.6	100	89	191	100	100	0.5
Positive Control	1.6	-	2.3	-	0.7	7	288	7	4	0.3	41.1
PANA											
0.5 $\mu\text{g/ml}$	3.1	-	11.2	-	8.1	84	68	214	112	94	0.3
5.0 $\mu\text{g/ml}$	2.7	-	8.6	-	5.9	61	5	300	157	95	0.02
10.0 $\mu\text{g/ml}$	2.6	-	10.9	-	8.3	86	50	202	105	122	0.3
25.0 $\mu\text{g/ml}$	3.4	-	12.4	-	9.0	93	57	215	112	104	0.3
<u>Activation</u>											
Solvent Control	1.1	-	7.2	-	6.1	100	58	229	100	100	0.3
Positive Control	1.5	-	2.7	-	0.2	3	184	8	3	0.9	2.3
PANA											
0.005 $\mu\text{g/ml}$	3.3	-	17.8	-	14.5	246	8	56	69	170	0.1
0.01 $\mu\text{g/ml}$	3.1	-	15.6	-	12.5	212	3	58	72	152	0.05
0.05 $\mu\text{g/ml}$	2.8	-	6.5	-	3.7	62	32	70	86	54	0.5
0.1 $\mu\text{g/ml}$	0.9	-	1.7	-	0.8	14	6	36	44	6	0.2

Unscheduled DNA Synthesis

A second component of the in vitro mammalian cell assay system utilized the human diploid WI-38 strain of cells. This cell strain, obtained from human embryonic lung, was used to measure test chemical-induced DNA repair in cells not undergoing scheduled (S phase) DNA synthesis.

Normal DNA synthesis occurs in the S phase of the cell cycle with little or no synthesis occurring in any of the other phases (G_0 , G_1 , G_2 or M). The detection of significant DNA synthesis during these stages (UDS) is indicative of the stimulation of repair enzyme systems. Exposure of WI-38 cells to various forms of radiation or to chemicals known to be mutagenic or carcinogenic has resulted in the stimulation of UDS (Stich and Laishes, 1973).

The detection of UDS in WI-38 cells involved exposure of the cells to the test chemical followed by the addition of $^3\text{H-TdR}$ to the culture. If DNA damage has been induced, the $^3\text{H-TdR}$ will be incorporated during the repair of the DNA. This incorporation can be detected by scintillation counting.

In these tests, there was no response to UDMH (Table 8) in nonactivation tests. The data from activation studies indicated a positive effect (a greater than 200% increase in the Activity Index). However, the effect was dose-related only over the two lower dose levels and dropped off at 1.0 $\mu\text{l}/\text{ml}$. Cellular toxicity at the high dose might explain the response in this stimulation.

TABLE 8. MEASUREMENT OF UDS IN WI-38 CELLS TREATED WITH UDMH

<u>Test</u>	<u>Concentration ($\mu\text{l}/\text{ml}$)</u>	<u>DNA(μg)</u>	<u>DPM</u>	<u>Activity Index^a</u>	<u>Percent of Control^b</u>
<u>Nonactivation</u>					
Solvent Control	-	9.02	76	8.4	-
Positive Control	MNNG (10 $\mu\text{g}/\text{ml}$)	1.76	86	48.9	582
UDMH	0.1	9.26	84	7.7	92
	0.5	4.45	54	12.1	144
	1.0	7.94	71	10.5	125
<u>Activation</u>					
Solvent Control		13.64	78	5.7	-
Positive Control	2-AAF (30 $\mu\text{g}/\text{ml}$)	2.45	60	24.5	430
UDMH	0.1	6.54	78	11.9	209
	0.5	8.88	120	13.5	237
	1.0	10.88	115	10.6	186

^aActivity Index = DPM/ μg DNA (DPM = Disintegrations/minute)

^bPercent of Control = $\frac{\text{Activity Index Treated}}{\text{Activity Index Control}} \times 100$

MH was clearly negative (Table 9). The sample cells at the high dose level (activation assay) were lost by breakage in the centrifuge. However, there was no indication of a trend or any activity in an equivalent dose in nonactivation tests.

TABLE 9. MEASUREMENT OF UDS IN WI-38 CELLS TREATED WITH MH

<u>Test</u>	<u>Concentration (μl/ml)</u>	<u>DNA(μg)</u>	<u>DPM</u>	<u>Activity Index^a</u>	<u>Percent of Control^b</u>
<u>Nonactivation</u>					
Solvent Control	-	9.02	76	8.4	-
Positive Control	MNNG (10 μ g/ml)	1.76	86	48.9	582
MH	0.1	9.64	102	10.6	126
	0.5	16.88	83	4.9	58
	1.0	9.25	102	10.4	124
<u>Activation</u>					
Solvent Control	-	13.64	78	5.7	-
Positive Control	2AAF (30 μ g/ml)	2.45	60	24.5	430
MH	0.1	22.56	145	6.4	112
	0.5	14.85	79	5.3	93
	1.0	Sample Lost			

^aActivity Index = DPM/ μ g DNA (DPM = Disintegrations/minute)

^bPercent of Control = $\frac{\text{Activity Index Treated}}{\text{Activity Index Control}} \times 100$

Data from nonactivation tests with PANA (Table 10) show a weak positive response at 50 μ g/ml. The next higher dose used, 100 μ g/ml, was toxic for the cells and a measurement of UDS could not be made. The data from the activation tests were negative. The positive results might have been aberrant since they were not observed in the activation test, or the activation system may have detoxified or quenched the potential activity observed in nonactivation tests.

TABLE 10. MEASUREMENT OF UDS IN WI-38 CELLS TREATED WITH PANA

<u>Test</u>	<u>Concentration (μg/ml)</u>	<u>DNA(μg)</u>	<u>DPM</u>	<u>Activity Index^a</u>	<u>Percent of Control^b</u>
<u>Nonactivation</u>					
Solvent Control	-	9.02	76	8.4	-
Positive Control	MNNG (10 μ g/ml)	1.76	86	48.9	582
PANA	10	6.00	79	13.2	157
	50	3.51	67	19.1	227
	100	Toxic	—	—	—
<u>Activation</u>					
Solvent Control	-	13.64	78	5.7	-
Positive Control	2AAF (30 μ g/ml)	2.45	60	24.5	430
PANA	5	19.48	80	4.1	72
	10	9.34	55	5.9	104
	50	18.24	104	5.7	100

^aActivity Index = DPM/ μ g DNA (DPM = Disintegrations/minute)

^bPercent of Control = $\frac{\text{Activity Index Treated}}{\text{Activity Index Control}} \times 100$

A repeat test for UDS (Trial II, Table 11) also showed activity in nonactivation tests. One point in the activation tests also appeared positive. The lack of a clear dose-related response reduced confidence in the effect but indicates further testing is warranted.

TABLE 11. REPEAT MEASUREMENT OF UDS IN WI-38 CELLS
TREATED WITH PANA

Test	Concentration (μ g/ml)	DNA(μ g)	DPM	Activity Index ^a	Percent of Control ^b
<u>Nonactivation</u>					
Solvent Control	-	42	162	3.83	-
Positive Control	MNNG (10 μ g/ml)	34	460	13.70	358
	PANA 5	32	439	13.90	363
	PANA 10	43	297	6.90	180
	PANA 50	45	514	11.40	296
<u>Activation</u>					
Solvent Control	-	44	153	3.51	-
Positive Control	2AAF (30 μ g/ml)	38	518	13.74	391
	PANA 5	42	219	5.24	149
	PANA 10	34	459	13.50	385
	PANA 50	45	184	4.06	116

^aSee Table 10.

^bPercent of Control = $\frac{\text{Activity Index Treated}}{\text{Activity Index Control}} \times 100$

Dominant Lethal Assay

This assay was designed to determine the ability of a compound to induce genetic damage to the germ cells of treated male mice and rats leading to fetal wastage. Chromosome aberrations, including breaks, rearrangements, and deletions, are believed to produce the dominant lethality. Male mice and rats were exposed to several dose levels of the test compound for five days and then sequentially mated to two virgin untreated females each week over the period of spermatogenesis. At mid-pregnancy the females were killed and scored with respect to the number of living and dead implants as well as to the level of fertility. These results were then compared to data from control animals.

Dominant lethality, which is indicated by a high percentage of dead implants to total implants was not demonstrated by data from UDMH, MH or PANA (Tables 12, 13 and 15) administered to mice or by MH administered to rats (Table 14). In comparison, the positive control compound TEM demonstrated a clear dominant lethal effect in mice during weeks 1 through 3 and in rats during weeks 1 through 5.

Isolated instances of apparent significant dominant lethality did occur among the experimental data. They were not associated with dose-related trends and had dead implant/total input ratios that fell within the range of all negative controls (e.g., PANA intermediate dose, week 6; MH, rats week 7).

TABLE 12. COMPOUND UDMH, STUDY SUBACUTE/MICE

Week	Dead Implants/Total Implants				Positive Control
	Negative Control	Dose Level 1.25 mg/kg	Dose Level 4.20 mg/kg	Dose Level 12.50 mg/kg	
1	0/ 67 = 0.0	0/ 47 = 0.0	0/ 75 = 0.0	1/ 37 = 0.03	7/ 16 = 0.44
2	7/155 = 0.05	1/ 56 = 0.02	4/ 70 = 0.06	4/ 92 = 0.04	39/ 50 = 0.78**
3	6/133 = 0.05	2/ 71 = 0.03	1/ 75 = 0.01	1/ 88 = 0.01	20/ 46 = 0.43**
4	10/156 = 0.06	1/ 80 = 0.01	9/182 = 0.05	8/ 69 = 0.12	8/160 = 0.05
5	0/ 12 = 0.0	2/ 39 = 0.05	4/127 = 0.03	2/116 = 0.02	0/ 12 = 0.0
6	4/ 97 = 0.04	6/127 = 0.05	10/129 = 0.08	6/140 = 0.04	7/183 = 0.04
7	6/110 = 0.05	5/ 48 = 0.10	10/146 = 0.07	6/112 = 0.05	4/117 = 0.03
8	4/ 77 = 0.05	8/ 57 = 0.14	5/ 87 = 0.06	5/ 59 = 0.08	1/132 = 0.01

Statistically significant increase over control values:

**Significant at P less than 0.01

TABLE 13. COMPOUND MH, STUDY SUBACTUE/MICE

Week	Dead Implants/Total Implants				Positive Control
	Negative Control	Dose Level 0.26 mg/kg	Dose Level 0.86 mg/kg	Dose Level 2.60 mg/kg	
1	0/ 67 = 0.0	0/ 35 = 0.0	0/ 58 = 0.0	0/ 46 = 0.0	7/ 16 = 0.44
2	7/155 = 0.05	6/122 = 0.05	7/113 = 0.06	0/ 51 = 0.0	39/ 50 = 0.78**
3	6/133 = 0.05	0/116 = 0.0	4/115 = 0.03	5/117 = 0.04	20/ 46 = 0.43**
4	10/156 = 0.06	5/ 99 = 0.05	11/131 = 0.08	5/149 = 0.03	8/160 = 0.05
5	4/ 97 = 0.04	5/148 = 0.03	5/153 = 0.03	5/115 = 0.04	7/183 = 0.04
6	6/110 = 0.05	1/ 48 = 0.02	8/156 = 0.05	2/ 63 = 0.03	4/117 = 0.03
7	4/ 77 = 0.05	6/107 = 0.06	4/131 = 0.03	6/ 96 = 0.06	1/132 = 0.01

Statistically significant increase over control values:

**Significant at P less than 0.01

TABLE 14. COMPOUND MH, STUDY SUBACUTE/RATS

Week	Dead Implants/Total Implants				
	Negative Control	Dose Level 0.215 mg/kg	Dose Level 0.720 mg/kg	Dose Level 2.150 mg/kg	Positive Control
1	6/ 94 = 0.06	4/ 67 = 0.06	3/ 69 = 0.04	2/ 93 = 0.02	15/ 97 = 0.15
2	1/104 = 0.01	7/147 = 0.05	1/148 = 0.01	2/171 = 0.01	3/ 70 = 0.04*
3	8/100 = 0.08	4/115 = 0.03	6/176 = 0.03	0/174 = 0.0*	46/ 47 = 0.98**
4	10/188 = 0.05	8/207 = 0.04	7/144 = 0.05	16/212 = 0.08	28/ 30 = 0.93**
5	7/148 = 0.05	4/148 = 0.03	12/151 = 0.08	19/180 = 0.11	85/128 = 0.66**
6	13/178 = 0.07	5/146 = 0.03	5/191 = 0.03	10/182 = 0.05	23/141 = 0.16*
7	0/255 = 0.0	10/167 = 0.06**	8/188 = 0.04	6/236 = 0.03*	5/144 = 0.03**

Statistically significant increase over control values:

*Significant at P less than 0.05

**Significant at P less than 0.01

TABLE 15. COMPOUND PANA, STUDY SUBACUTE/MICE

Week	Dead Implants/Total Implants				
	Negative Control	Dose Level 50.0 mg/kg	Dose Level 166.0 mg/kg	Dose Level 500.0 mg/kg	Positive Control
1	0/ 72 = 0.0	1/ 45 = 0.02	1/ 64 = 0.02	0/ 34 = 0.0	7/ 16 = 0.44*
2	3/118 = 0.03	3/126 = 0.02	5/ 77 = 0.06	5/135 = 0.04	39/ 50 = 0.78**
3	7/117 = 0.06	1/ 75 = 0.01	0/ 84 = 0.0	5/ 97 = 0.05	20/ 46 = 0.43**
4	4/138 = 0.03	5/ 81 = 0.05	5/ 61 = 0.08	1/106 = 0.01	8/160 = 0.05
5	4/ 50 = 0.07	5/106 = 0.05	1/ 44 = 0.02	3/ 60 = 0.05	0/ 12 = 0.0
6	4/204 = 0.02	3/143 = 0.02	9/148 = 0.06*	4/142 = 0.03	7/183 = 0.04
7	5/139 = 0.04	1/ 28 = 0.04	6/112 = 0.05	7/116 = 0.06	4/117 = 0.03
8	7/135 = 0.05	8/125 = 0.06	4/ 74 = 0.05	2/ 68 = 0.03	1/132 = 0.01

Statistically significant increase over control values:

*Significant at P less than 0.05

**Significant at P less than 0.01

CONCLUSIONS

The accumulated data from the test battery suggest that UDMH is metabolically activated *in vitro* (liver microsomes) to an intermediate that possesses genetic activity. The forward mutation induction of TK-/- mutants was the only strong response obtained, but supported data from certain microbial tests (TA-98) and the DNA repair test in WI-38 cells. These data, examined together, were consistent in establishing a trend of activity which was not sufficient to indicate activity if examined as an isolated study. The negative results from the dominant lethal assay also fit the pattern shown by hydrazine which is mutagenic in microbial tests but not active in the dominant lethal assay (Röhrbörn et al., 1972). The chemical reactivity of the active molecule may be responsible for the lack of response in this test since other methylating agents (MNNG) and nonfunctional alkylating agents (β -propiolactone) are also negative in dominant lethal tests but mutagenic in a wide range of *in vitro* systems. Based on correlation data collected in the *in vitro* test systems, it can be concluded that UDMH exhibits biological activities consistent with most known mutagens and carcinogens.

MH was mutagenic in activation, microbial reversion tests if the tests were conducted as suspension tests but not if conducted as standard plate tests. This differential activity is similar to the type of results obtained with dimethyl- and diethyl-nitrosamines. Except for the mutagenicity of MH in tests with S. typhimurium TA-1535, there were no indications of genetic activity for MH in any of the other tests conducted as part of this evaluation.

Except for the nonactivation results with PANA that appeared to stimulate UDS in WI-38 cells, the data from the other test systems were negative. Although the results would normally be considered aberrant, it cannot be completely dismissed without additional observations. The overall conclusions from the accumulated data were that there was no consistent pattern of responses indicative of genetic activity by PANA and that the compound did not exhibit biological activity consistent with the known mutagens and carcinogens. However, the reproducibility of the UDS response indicates the need for further investigation.

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OPEN FORUM

DR. WINSTEAD (National Academy of Sciences): I'd like to direct a question and a comment to Dr. Weisburger. First the comment; while showing your slides, you discussed the risk of lung cancer and if I understood correctly, you made the statement that nonsmokers had zero risk. I assume you must mean zero increased risk. I would like to ask you to comment on the scientific basis of labelling benzene as a leukemogen.

DR. WEISBURGER (Naylor Dana Institute for Disease Prevention): If you look at patients with respiratory tract cancer, lung cancer, you will find that 95% of them were heavy, long time smokers. There are also a small number of patients with bronchogenic carcinoma who apparently have never smoked. The reason is what we call cryptogenic. We don't know what caused their cancer. My colleague, Dr. Winter, who is an epidemiologist published an article entitled "Microepidemiology" in Preventive Medicine. He described one case, that of Dr. Manfred Karnovsky, who was his colleague and friend at the Sloan-Kettering Institute. Karnovsky had never smoked. He died in the late fifties of oat cell carcinoma. This was a bit amazing. Number one, the histological classification of the tumor was different. And secondly, since he had never smoked, what caused it? In a chapter of a book I wrote, I said that all cancers had causes. Our job is to discover causes. It turns out that Karnovsky was an officer at Edgewood during World War II where he was exposed to "war gases" including mustard. One has reason to believe that his cancer, oat cell carcinoma, was due to his chemical exposures during the year and a half he worked at Edgewood. Twenty years later his cancer came out. To summarize an answer to your question, lung cancer incidence is due in major part to smoking but we do also have exposures at coke ovens and other types of occupational exposure that account for a small but avoidable number to be sure. I hope the work we heard presented this morning will lead to conditions such that even coke oven workers and others in the industrial and chemical environment have zero risk. The environment should be designed to do that. We also need good pathology, oat cell carcinoma is different in causative relationships from bronchogenic carcinoma as some other pulmonary diseases are different. Your second question was about benzene. There are case reports in the literature, Dr. Winstead, about this. We need to know more of the genetic idiosyncrasy

where some individual may be more sensitive to leukemia development as a result of benzene exposure. Recently, I became aware that people working in the styrene and butadiene industries may also have a higher sensitivity. I have tried to experimentally cause leukemia in the laboratory in a very select animal model. I got anemias. I got all kinds of diseases but not leukemia.

DR. WINSTEAD: I'm well aware of your work. Some epidemiological data now is showing some of the same type things with other solvents as shown in the case histories for benzene which, of course, was in all instances mixed solvent exposures. Just recently NIOSH has labelled benzene as definitely a carcinogenic compound. Since you had mentioned benzene in your presentation, I was interested in what you thought of the scientific basis for the NIOSH position.

DR. WEISBURGER: Most case histories of benzene related leukemia, as you know, come from France and Italy with very few reported in this country. It's very important to look at this because as you know, 40% or 50% of the lead-free gasoline sold in America today is aromatic to keep the octane rating up.

DR. BRUSICK (Litton Industries, Inc.): I'd like to address just one comment to Dr. Weisburger's review of carcinogenesis. I think there is one group of materials that I would like to have him comment on. That is the steroids. This is becoming more important all the time, the potential for steroid carcinogenesis.

DR. WEISBURGER: That is, of course, very important since we are now doing a million women experiment on oral contraceptives. Steroids are not carcinogens in the classic sense. And that's why also, Dr. Brusick, as you will note, they are not mutagenic. We all have a certain hormonal balance which makes us males or females as the case may be. We are measuring these things in our laboratory now, pituitary, thyroid, and adrenal hormones, to see what the proper balance might be in a normal individual. And we found that hormonal balance, particularly of prolactin, the pituitary hormone, is a function of diet. We are beginning to have an understanding of some of the involvement of these hormones, say in breast cancer. We've begun the same program in prostate cancer. Hormones are not carcinogens as such. I have so stated in a review that I did on tests for chemical carcinogens. They are involved in cancer production only indirectly by

indirect mechanisms. Recently some of you may have heard test reports of women on oral contraceptives who developed "benign" liver tumors, which nonetheless were fatal because they hemorrhaged easily. These cases are rare. One has to ask the question, "Is the hormone simply enhancing or promoting that risk in these few women?" It's something that we don't know yet. I have asked also this question, "Has there been an antecedent hepatitis episode in these females?" The answer was no. Other than that, I don't think that we can call hormones carcinogenic. Nonetheless their imbalance as we age may lead to these cancers. Breast cancer, ovarian cancer, intramedial cancer, and prostate cancer in males, certainly are hormonal responses.

DR. BRUSICK: You wouldn't foresee the trend with the continued use of antifertility drugs in women for target organ carcinogenesis similar to what you are seeing now in lung cancer, that the incidence curve may start going up for breast cancer and so on in the next 20 years with the continued use of these drugs?

DR. WEISBURGER: Dr. Brusick, I've been always called an optimist. I will say this. I hope not since the use of these drugs is so prevalent. Recently in the medical field, it has been demonstrated in older ladies who were put on estrogen for various indications that there is a relationship between obesity, fat intake, and the occurrence of intramedial cancer. We are just now studying the endogenous hormones, not exogenous, and it could be that one has to be very careful. Now, as I said, the normal human female that's cycling properly has a 21-day on, 7-day off estrus cycle. It turns out that many of our medical brothers when we put women on estrogen, they don't cycle them anymore. They feel they are in menopause and it doesn't matter. I believe, and this is just a belief that I have, that it might matter and if they give any chemical, whether it is a drug or a hormonally active chemical, that it should be given in a cyclic manner to mimic the normal human cycle even though the cycle is gone. Maybe in this way the risk of cancer would be less. We have to do a lot more studies. This is 1976. We know more than 10 years ago. Ten years from now, we will know more yet. We have to do a lot more research.

DR. BACK (6570 Aerospace Medical Research Laboratory): I'd like to ask anybody on the panel if nutrition is a part of the oncogenic picture and if overeating in our country is part of the oncogenic picture possibly, what do you propose is the mechanism? Is it that as one is heavier and has more fatty tissue, that he has a higher uptake of certain chemicals that produce the oncogenic response? Most fat people also have fatty livers. Would you expect the mechanism is related to increased absorption into fat tissue of chemically active agents or not?

DR. WEISBURGER: I don't want to monopolize this situation because we have other important considerations but certainly obesity as such is associated with only two risk factors for early death, coronary heart disease on the one hand and intra-medial cancer within the cancer series. The other cancers are not associated with obesity as far as we can tell now. I couldn't present all of our data, but at our Institute we have several of the major risk factors, respiratory tract, digestive tract and endocrinial cancers, all organized along research lines. We are finding that people on a high fat diet, whether they are obese or not, have high bile acid output as I showed you, higher levels of prolactin, and lower levels of estrogen, all of these being indicative of certain risks. What we are saying is that fat translates to certain of these parameters. We were asked if the fat included or contained "carcinogens." We don't know. We have shown that some of these things had a "cocarcinogenic" promoting effect. We must ask then, "What is the carcinogen?" And that we don't know yet. But we don't really think it is associated with the fat. It must be something else.

DR. STEVEN LEWIS (Exxon Corporation): Dr. Weisburger, I'm sorry but I didn't exactly get the number mentioned for the percent benzene in gasoline.

DR. WEISBURGER: I'm not an expert but the aromatic fraction is up to 40% or more.

DR. LEWIS: I don't think so. I'm not aware of any gasoline blending operations that are using any more than about 3 or 4% benzene at the most in the United States. There are some European applications that are using more.

DR. WEISBURGER: In the new lead-free, high test gasoline?

DR. LEWIS: Well, Exxon is not, at least. And I'm not aware of any of the other major refiners that are blending at those concentrations.

DR. WEISBURGER: Well, you are the expert, not me.

DR. LEWIS: I have a question for Dr. Brusick. I wonder about some of the instances where the dose response relationship apparently fails in the microbiological tests and I'm concerned about some kind of testing mechanism to evaluate the true toxicity of those materials to the organisms. Maybe in my naivety I don't understand but would it be possible to test the wild strain, the revertant, in the presence of the concentrations of the carcinogens or mutagens you are studying to see if, in fact, they are toxic to the organism itself so as to somehow account for this situation where we see roughly the same number of mutants across hundred fold differences in test concentrations?

DR. BRUSICK: The way the bacterial tests are set up, you continually increase dose level until you see a slight, or at least a significant, drop at some of the higher dose levels in the number of revertants indicating that you are killing off some of the cells and, therefore, you have reached a point of toxicity. This is a qualitative test and not a quantitative test as such. You are really looking at qualitative or semi-qualitative results where you are just trying to use concentrations that would be high enough to give some toxicity but not kill off more than 50% of the total cells that have been exposed to the compound. And then you would drop back from that through a series of half log or log steps down. But if you can't see any activity going that high then you will probably not see any if you continue increasing your dose levels.

DR. LEWIS: It seemed like quite a jump from just seeing roughly equivalent counts, one particular case comes to mind, roughly two-fold increase in the number of mutants in the treatment groups all across a couple orders of magnitude of doses. It seems to me that it's a pretty good size jump from the raw data to assume that the lack of a dose response relationship is strictly due to the inherent toxicity of the treatment. What I'm asking is, are we likely to develop some more sensitive reflection of true toxicity apart from mutagenicity so that we can distinguish between the two?

DR. BRUSICK: I'm not sure we are both talking about the same thing. You can measure toxicity. You can make these tests completely quantitative and measure the number of surviving cells before treatment and then measure the mutation frequency as such, but in the case of the plate assays, you run concentration ranges so that if there is a dose response, you will see it. After you hit a certain level, we start killing the cells and you are not increasing the number of mutants in the population. To continue on increasing the dose will not show any effect. You have a reasonably good idea of what the surviving population is at this point so a two-fold increase normally really doesn't mean anything. You have to remember (I think this is something that is important) that the evaluation of this type of semi-quantitative data is strictly a subjective evaluation based on historical experience. The reason being that is you look at these numbers, they are all numerators of fractions. That is, the total population of cells exposed would be the denominator and then the number on the top would be the number of reverse mutations observed. So the denominator in the cases where there is very low or no toxicity is in the range of a billion. So you are looking at numerators of 20 to 25 and denominators of a billion plus or minus a few percent. Therefore, 25 over a billion is no different than 15 over a billion generally. So you have to use other criteria. You have to use enough doses to demonstrate very clearly a dose response over at least 3 or 4 concentrations and some quantitative level greater than the background which can be fixed at no less than $2\frac{1}{2}$ times or 3 times.

DR. FRIESS (Naval Medical Research Institute): To keep Dr. Brusick working, there is a game which is played about every six months in terms of correlation between mutagenic potential with the battery screen and carcinogenic induction in an animal model. The last I remember about a year ago, it was approaching something like 90% correlation for about 200 chemicals that had been tested. What is the box score now? Do you happen to know?

DR. BRUSICK: I have to say that these have not been selected necessarily at random and the tests have not been, in all cases, conducted blind. So if we assume that there is a slight amount of bias in selecting chemicals and then doing the tests coded, it is running around 90%. But what really has to be done and what is being done now on a more systematic level is to look at class by class. For certain types of chemicals, the predictability is 99%, for others it's around 50% or maybe a little bit more.

Total overall correlation is running up close to 90%. But you have to examine the classes of chemicals tested. With some classes, these types of tests work very, very nicely. On others, it doesn't work as well. This may be a function of the test. There are a lot of factors involved that we don't understand and we don't even know how well the animal model is doing in terms of predicting with respect to basing another model system of that model system. But in general, of the assays that we have been looking at, that is, salmonella mutagenicity, tissue culture assays for mutations, DNA repair or transformation, all of them roughly over a fairly wide range of chemicals give about 90% correlation.

DR. FRIESS: Would we be wise to recast our thinking and put our questions in the terms of classes from now on?

DR BRUSICK: I think that would be a much better way of doing it.

DR. A. THOMAS (6570 Aerospace Medical Research Laboratory): Dr. Brusick, would it make a difference when you do the material test whether you use mouse liver homogenate or rat liver homogenate?

DR. BRUSICK: For some chemicals it does make a difference.

DR. THOMAS: Do you do both, or just arbitrarily pick one?

DR. BRUSICK: Normally, we try to optimize the system. Convention has it today that rat liver from animals that have been induced with a microsome inducer to enhance activity are typically used. Depending upon the preliminary toxicity data or other information that one can obtain, you may select other types of tissue sources for activation but routinely rat liver is used.

DR. HENDERSON (Olin Corporation): One for Dr. Brusick and one for Dr. Weisburger. Dr. Brusick, did you look at the impurities in the UDMH? Did you look for nitrosamines? Did you know what your impurity was? The same for the MMH in terms of diazo compounds? For Dr. Weisburger, in looking at respiratory effects of occupational exposure including lung cancer, we always look at smoking history. I'm curious whether in the county studies where they saw an increased incidence of liver effects, they also looked at the alcohol consumption history?

DR. BRUSICK: The answer for the question to me is no, we have not looked at the impurities. These compounds were tested under contract and were supplied by the contractor. Based upon these data, it would be beneficial to go back to the compounds and look at their purities. But I don't know what the purity of the compounds that were supplied was.

DR. WEISBURGER: The study was done by Hoover and Froumani and associates and I don't know whether they did look at personal habits, but you would think that they might not be dramatically different although perhaps there is an urban/rural difference in drinking habits. This is preliminary. It's simply something that we ought to consider in our thinking. I believe that lifestyle may be much the most important thing to the public at large but at the same time, sir, we urge you in industry and the Armed Services here who control a great deal of activities where chemicals are used, that you be very careful and use the technology that we now have to do prospective and retrospective studies of the type my two colleagues here have done in relation to the coke oven workers. That this be extended, that we use animal tests as done by Dr. MacEwen, the whole battery of things. You know, this is the bicentennial year of our Republic. It is also the bicentennial of Percival Pott and I think we know a lot today to prevent disease.

DR. BACK: Dr. Henderson asked the question about purity of the MMH and the UDMH used in the study. To the best of our knowledge, the UDMH was as free of n,n-nitrosodimethylamine as we could get it. It was the cleanest batch we had by mass spectrometry. I think the MMH may have been contaminated with the diazo compound. Now to what extent we don't know because it changes from batch to batch and even from bottle to bottle depending upon how much nitrogen has been bubbled through it. But it was as pure as we could make it. I might also point out that Dr. Brusick used n-nitrosodimethylamine as a positive control on this study. If we had any great amount of DMNA in the unsymmetrical dimethylhydrazine, I think it should have been as potent as the control.

COLONEL STEINBERG (Department of the Army, Office of the Surgeon General): Dr. Brusick, based on what you said, would you then recommend that one ought to run an enzyme induction screen on a particular compound before putting it into your mutagenic screen, and if so, which enzyme induction screen ought one to approach with this?

DR. BRUSICK: You mean to see if it is a microsome inducer?

COLONEL STEINBERG: Well, if you're going to run an assay in selecting an animal. The question earlier was, should one use the mouse or the rat or something else for screening tests? It would seem that one should run a test to see if that particular animal in fact does metabolize the compound before you put it into your screening system.

DR. BRUSICK: Yes, I'd say that in general we have not found for most chemicals a qualitative difference between the rodent species. We have not obtained chemicals where there is none versus a high level of enzyme induction. There are some quantitative differences, however. One may be lower than the other. And in that case, I think that your comment would then be absolutely correct, that it would be very beneficial to screen several species to find out if there are quantitative differences and maybe select the one that does have the highest level to do your screening with. It's certainly possible and it's something that can be done relatively easily, or to just set up a battery of tests including a number of species to begin with.

DR. STERNER (University of California, Irvine): Before we close the session, I would just like to ask the panel the following. We saw the presentation of data of nearly 100% tumors in one experimental species. How do you interpret the relatively low human incidence of disease?

DR. WEISBURGER: Dr. Sterner, with experimental animals you control everything; diet, ages starting, species, strain, and above all, you control the dose. We do, in carcinogenicity studies, usually do preliminary toxicology, select a high dose, and thus, we can get 100% tumors. Now if we were to mimic human situations, we would get lower doses and the important thing to realize is that some chemical carcinogens that are quite active at high dose levels have a very steep dose response curve so that if you drop the dose by half, you will find the tumor incidence dropping. Others have a very shallow curve where you may have activity over a range of 10^3 depending on many factors such as rate of absorption and metabolism. Fortunately for all of us, the human is not exposed to high levels. Even if a fellow smokes 2 packs of cigarettes a day, it isn't as much as what we put into our animals. Otherwise the data we have on human cancer incidence would be a lot higher. Our aim is to lower the environmental dose as much as we can for man.

DR. MITTMAN (City of Hope National Medical Center): There are obviously differences between individuals in terms of their susceptibility to all of the effects that we were talking about. Perhaps it is equivalent to the species differences which are repeated over and over again in the laboratory when one strain of animal may turn up with 100% and another with 0 or a small percent of tumors when given the same challenge dose. The challenge, or one of the challenges that we have taken, is to try to begin to sort out these individual susceptibility differences and in that way perhaps be able to predict which individual should not be exposed to these kinds of hazards.

DR. REDMOND (University of Pittsburgh): I'd like to point out, too, that in one of the tables which I showed during my presentation today of the group of workers who were most highly exposed to coal tar at the top of the ovens, some 27% died of lung cancer over an 18 year observation period. This being in the presence of other causes of death as well so that for the highly exposed, one is not talking about a small level of risk even in the human data.

DR. MAC EWEN (University of California, Irvine): I want to reiterate what Dr. Redmond just said. The response to the highest level of exposure she reported was not unlike both of the strains of mice. The rats were more unique in that essentially 100% of the exposed animals showed that particular tumor, but in the mice, we had approximately a 3 to 11 fold increase in tumor incidence. Now that's the same range as she was reporting, a 5 to 15 fold risk increase. In the one particular species of mouse, we found something like a 50% incidence of tumors. If you subtract the normal tumors, this was an increase over the normal of perhaps 40%. She has reported approximately 20-some percent cancer death in men exposed long-term to coke ovens topside. I don't think those are different kinds of data. I'd also like to point out that the exposure levels used in our experiments weren't really unrealistic doses. The 10 mg/m³ exposure levels we used are probably lower than some that coke oven workers were exposed to topside before air pollution controls were installed. The proposed OSHA exposure limit is something like 150 µg/m³ of the benzene soluble fraction of total particulate matter. This is similar to the 0.2 mg/m³ exposures we conducted in the continuous exposure experiment. When we exposed animals to these aerosol levels, we didn't see any increased incidence of skin tumors above the control level of spontaneous tumors.

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SESSION II

INHALATION TOXICOLOGY

Chairman

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THE CURRENT STATE OF CHAMBER DESIGN AND INHALATION
TOXICOLOGY INSTRUMENTATION

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From the year 1914 when Professor Haber in Germany recognized and applied the concept of $C_t = K$ (Haber, 1924) for chemical warfare purposes to the year 1976 when inhalation toxicology experiments are conducted mainly for the understanding of the health effects of airborne industrial toxicants and environmental pollutants, inhalation toxicology has become a branch of biological science which requires a considerable amount of technological know-how with respect to exposure chambers and instrumentation.

EXPOSURE CHAMBERS

Most inhalation experiments are conducted in exposure chambers in which the animal receives a whole body exposure. Several comprehensive reviews (Fraser et al., 1959; Drew and Laskin, 1973) on exposure chambers showed that the size and shape of chambers can vary from a small cylindrical glass battery jar to a spherical plastic bubble to a cubical room. There are also specialized chambers for studying inhalation toxicity of airborne toxicants at low atmospheric pressure, simulating a space environment (Thomas, 1965), and in high pressure simulating underwater conditions (Rose et al., 1970). But the types of chambers most commonly used for toxicity studies are the 3 foot cubicals with pyramidal top and bottom. Each chamber can accommodate 50 to 100 rats, and several chambers can be installed in a large room. The animals are loaded into the exposure cages each morning and then returned to a regular animal holding room after the exposure. This type of chamber and laboratory facilities have been used satisfactorily for subacute and chronic exposure studies for many years. However, recent emphasis on the use of large animal populations for carcinogenesis studies, and the emphasis on proper

animal housing conditions have led to some critical changes in the design of today's exposure chambers. In general, the exposure chamber has to be large enough to accommodate an animal sample size sufficiently large to permit a valid statistical evaluation of the experimental results. For example, Table 1 shows the average number of animals needed for detecting a significant increase in the incidence of an event (tumors, anomalies, etc.) as a function of the background incidence (control) and the expected incidence (treatment).

TABLE 1. AVERAGE NUMBER OF ANIMALS NEEDED TO DETECT A SIGNIFICANT INCREASE IN THE INCIDENCE OF AN EVENT (TUMORS, ANOMALIES, ETC.) AS A FUNCTION OF THE BACKGROUND INCIDENCE (CONTROL) AND EXPECTED INCIDENCE (TREATMENT) USING THE FISHER EXACT PROBABILITY TEST $P < 0.05$

Back-ground Inci-dence, %	Expected Increase in Incidence, %					
	0.01	0.1	1	3	5	10
0	46,000,000 ¹	460,000	4,600	511	164	46
0.01	46,000,000	460,000	4,600	511	164	46
0.1	47,000,000	470,000	4,700	520	168	47
1	51,000,000	510,000	5,100	570	204	51
5	77,000,000	770,000	7,700	856	304	77
10	100,000,000	1,000,000	10,000	1,100	400	100
20	148,000,000	1,480,000	14,800	1,644	592	148
25	160,000,000	1,600,000	16,000	1,840	664	166

¹Number of animals needed in each category, controls as well as the treated group.

These numbers were calculated using the Fisher Exact Probability Test (Siegel, 1956). As indicated, if the background incidence of a certain type of tumor in a certain species is 1%, a population of 204 animals would be used, so that an observation can be considered to be significantly higher than that of the control at the 95% confidence level. When both sexes are used, the total number of animals will be 408. If this detection limit is accepted for an inhalation carcinogenesis study, an exposure chamber would have to be big enough to hold this many animals under acceptable housing conditions. According to

good laboratory practice for inhalation toxicology, the total space occupied by the animals should not be more than 5% of the volume of the chamber. This is desirable in order to avoid severe losses in agent concentration with the chamber (MacFarland, 1976). Consequently, the theoretical minimum size of the chamber suitable for exposing 400 rats, each weighing 300 grams, would be approximately 2400 liters (\approx a 4 foot cubical). Under such exposure conditions, the experimental animals have to be removed from the exposure chamber and returned to regular housing cages. The daily handling of hundreds of animals will require a tremendous amount of time and labor. Such an operating situation would be unacceptable both from a time and cost point of view. Therefore, there is a tendency to house the animals in situ in the chamber. However, according to the guidelines for the care and use of laboratory animals (U.S. Department of Health, Education and Welfare, 1974), the recommended floor area for a 300 gram rat is 40 square inches. Based on this dimension, a chamber of approximately 14,500 liters (\approx an 8 foot cubical) will be required to accommodate 400 to 500 rats. For these reasons, the inhalation facilities recently built by the Dow Chemical Company and those currently being built by the International Research and Development Corporation (IRDC) have been designed according to this aforementioned rationale.

The Dow exposure chamber (Figure 1) consists of an upper room for preparation of the experimental atmosphere, and a lower room for exposing and housing the animals. The bottom of the chamber is level instead of pyramidal in shape. This flat bottom construction makes it easier to move the animal holding racks around as well as in and out of the chamber. There are four chamber exhaust openings located at each corner of the floor of the chamber instead of the conventional single exhaust at the center of the pyramidal bottom.

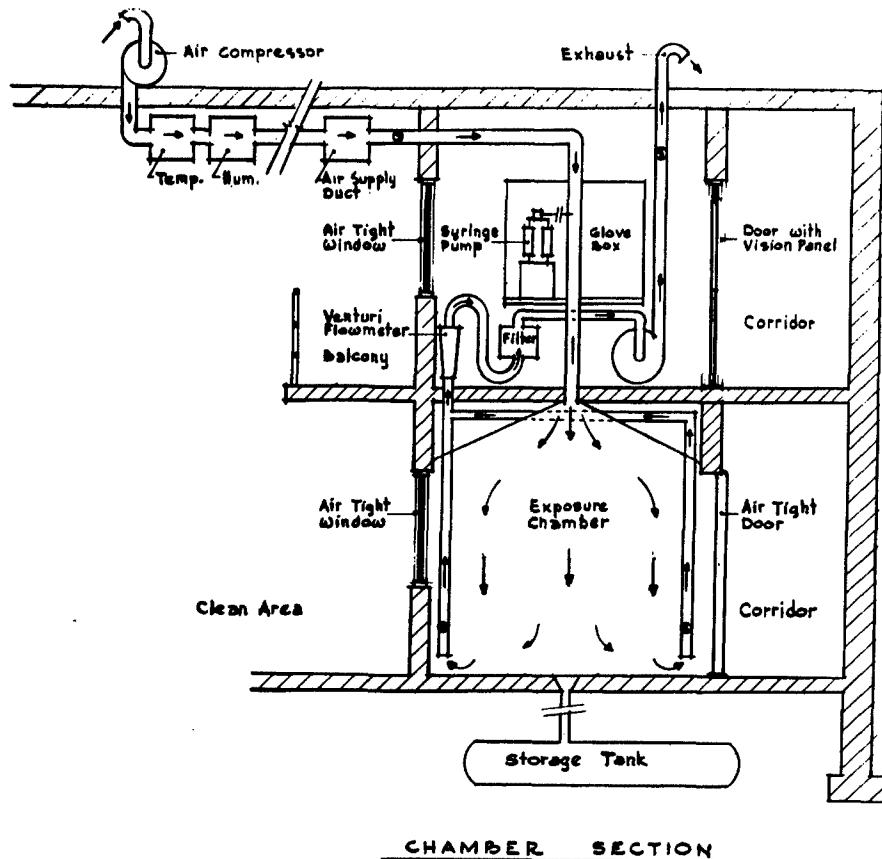


Figure 1.
Schmatic
Diagram of
the Dow
exposure
chamber.

The IRDC exposure chamber (Figure 2) consists of a conveniently reachable large glove box on the top of the exposure chamber for the preparation of the experimental atmosphere and an adjoining animal holding room separated by a door. The two racks of animals can be either kept within the exposure chamber or be rolled out of the chamber into the adjoining room for easier access for carrying out animal care activities. For exhausting the chamber atmosphere, there are two horizontal exhaust pipes located at floor level along two sides of the chamber. There are exhaust holes drilled on the exhaust pipe for even rate of exhaustion.

The distribution of the airborne toxicant in the chamber atmosphere was observed to be satisfactory in either "four corners" or "parallel holes" arrangements.

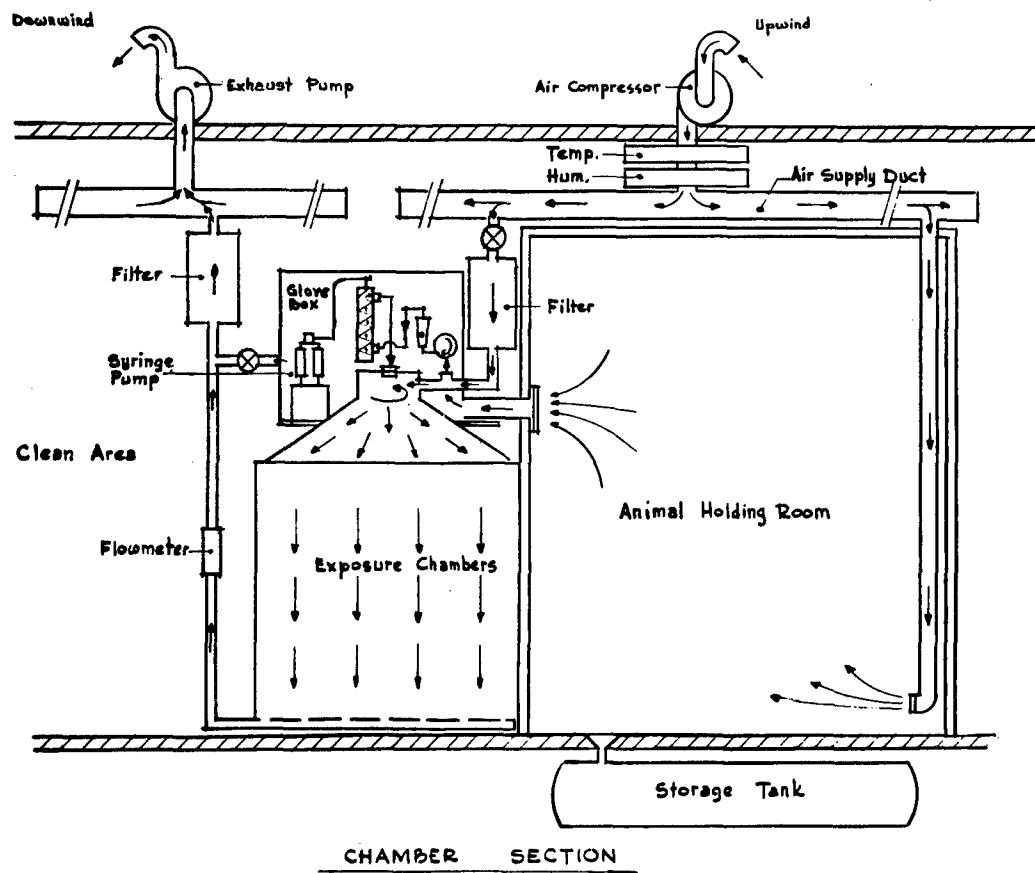


Figure 2. Schematic diagram of the IRDC exposure chamber.

The design of the chamber and its installation in a laboratory is also influenced considerably by the promulgation of the Occupational Safety and Health Administration (OSHA) standard for handling carcinogens (Federal Register, 1973). In this standard, considerable emphasis was placed on the design of the work place where carcinogens may be encountered. There are definite distinctions between the "controlled area" or regulated area as opposed to the unregulated area. There are also certain requirements for proper ventilation, clean-up, and waste disposal in the "controlled area."

In addition of the OSHA standards, the forthcoming promulgation of the guidelines for "good laboratory practices for non-clinical laboratory studies" by the Food and Drug Administration (FDA) will also influence the future design of facilities for inhalation toxicology. For example, the recommendation on conducting different research functions in separate areas and the separation of treated animal groups, etc., should be incorporated into the chamber design. In this respect, the floor plans of both the Dow and the IRDC inhalation laboratory (Figures 3 and 4) have reflected the application of the idea of separating the locations of different research functions - exposure, animal holding and experimental atmosphere generation. Furthermore, each exposure unit can function independently from the other units, thereby eliminating the possibility of cross-contamination of chemicals, as well as reducing the possibility of spreading spontaneous disease between treatment groups.

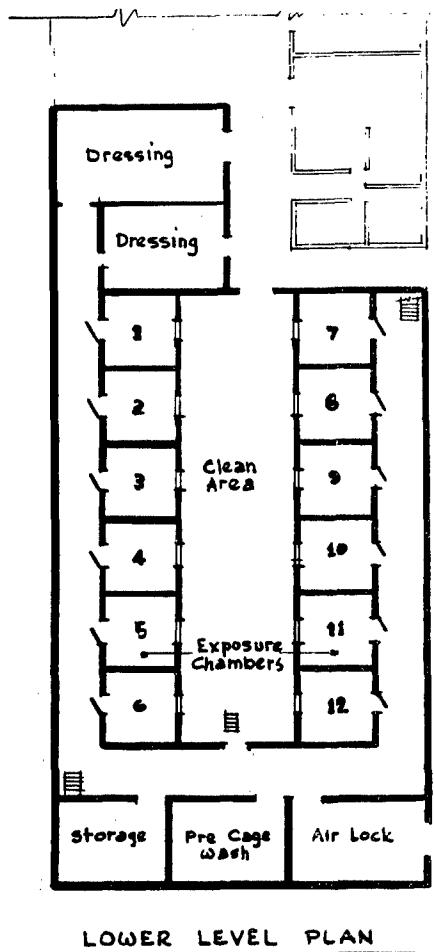


Figure 3. Floor plan of the Dow inhalation toxicology laboratory.

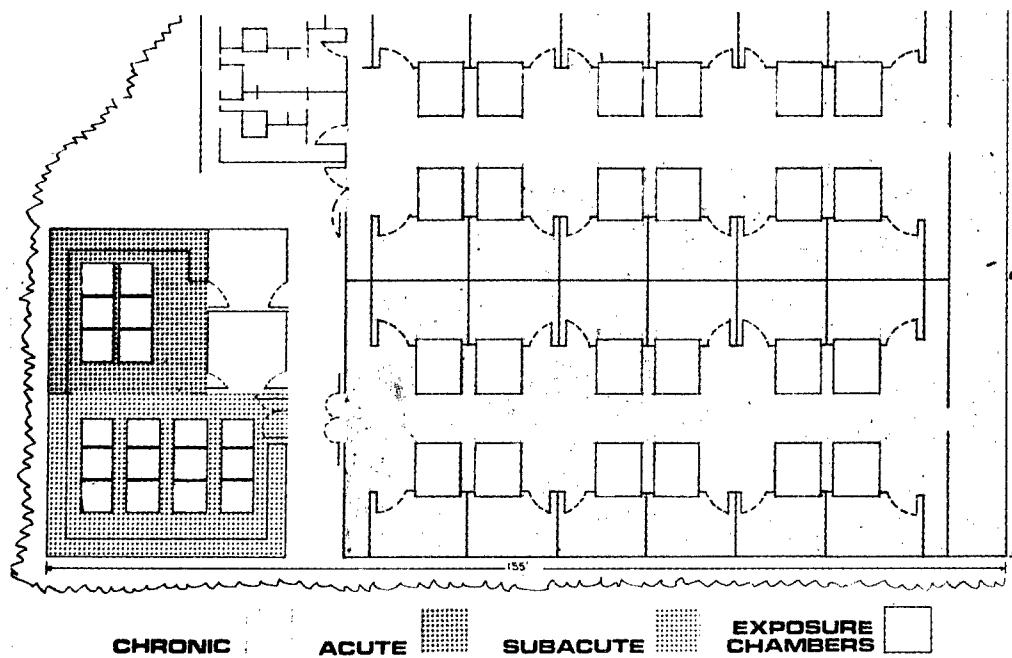


Figure 4. Floor plan of the IRDC inhalation toxicology laboratory.

In conclusion, the current state of chamber design is to apply the concept of "one chamber - one room - one project" and to achieve the performance of separate experimental functions in an integrated manner. The chamber should be capable of accommodating a large number of animals, should meet the requirements of the animal care guideline; and should meet the requirements for safe handling of highly hazardous compounds including carcinogens.

INSTRUMENTATION

Inhalation toxicology instrumentation encompasses various types of masks and helmets for head only dosimetric exposures; various types of apparatus for generating gaseous, vapor, aerosol or dust experimental atmospheres; and various physical and electronic instruments for measuring and monitoring the concentration of a compound in an experimental atmosphere. Most of the instruments, other than certain "gadgets" and commercially available monitoring equipments, are likely to be custom built within an individual laboratory for a specific need.

There are two kinds of apparatus worthy of mentioning, one for generating vapor and the other for dust chamber atmosphere. First, a modified version of the counter air-liquid current vaporization column (Carpenter et al., 1975) was found to be very effective for vaporizing a liquid mixture of different or high boiling points. Second, a motor driven chain-dispensing dust feeder (Marple et al., 1976) may be a better dust generator for dust inhalation studies.

It is anticipated many new types of apparatus and inhalation toxicology instruments will be developed in the future to meet the need of the logarithmic growth on the demand for conducting inhalation toxicity studies.

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BIOCHEMICAL EVENTS ASSOCIATED WITH TOXIC LUNG DAMAGE

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During the last few years, lung biochemistry has developed rather fast and has now come fully into its own. Thanks to work done in several laboratories we already know a great deal about the energy metabolism of lung tissue, the biosynthesis of macromolecules, and the capability of the lung to handle endogenous and foreign agents (Witschi, 1975). Work is underway to unravel the molecular changes underlying abnormal and chronic pathologic conditions of the human lung, such as fibrosis and emphysema (Hance and Crystal, 1975). This development raises for toxicologists the question: what can a biochemically-oriented approach contribute to our understanding of toxic lung damage?

I want to talk about three things biochemistry can do: it can provide clues to the mode of action of toxic agents in the lung; it can help to identify and to follow abnormal events in the lung tissue; and it can help to quantitate what is going on. Biochemistry can probably yield much additional information but these are the three essential points I want to emphasize and illustrate.

One of the greatest challenges in toxicology is to understand the mechanism of action of a toxic agent in molecular terms. However, this is not an easy endeavor. The problem of how to correlate biochemical events with later cell damage has in no organ been more thoroughly and ingeniously examined than in liver. And yet, we are not capable of saying with any certainty which ones of the many biochemical changes we identify are indeed linked to cell death and which ones are only peripheral, irrelevant, and possibly transient disturbances.

The same is true for lung. A good example to illustrate this point is some recent work done with the herbicide paraquat. Theoretical considerations and a series of well-designed experiments led to the following hypothesis concerning its mechanism of action: in the lung, paraquat is reduced to its free radical with accompanying oxidation of NADPH. Damage to those cells which accumulate paraquat may then be a consequence of the production of oxygen radicals, followed by lipid peroxidation of biological membranes (Bus et al., 1975). Another possibility could be that a depletion of NADPH per se leads to cell death (Rose et al., 1976).

It has been pointed out that experiments with paraquat should always be accompanied by control experiments with diquat. The reason for this is that paraquat damages the lung, whereas diquat supposedly does not. There is another important difference between the two compounds: after intravenous injection of 40 mg/kg of either paraquat or diquat, the plasma disappearance curve for the two agents is practically identical. However, the tissue distribution is different; 24 hours after injection, the lung contains 10 times more paraquat than diquat. It is now established that the accumulation of paraquat in the lung is an active, energy-dependent mechanism, whereas diquat is not taken up actively (Rose et al., 1974).

We might, therefore, expect that any effect of paraquat upon pulmonary NADPH is greater than the effect of diquat. However, this is not at all so. Within a very short time after intravenous injection of either compound, the lungs become substantially depleted of NADPH and the ratio of NADPH/NADP falls below 50% of control values. For the next 24 hours, they stay around 50% for paraquat, whereas with diquat, a slight recovery seems to occur. The oxidation of NADPH to NADP is therefore approximately the same after both diquat and paraquat, although the latter accumulates 10 times more in lung than does diquat.

If we calculate total amounts of pyridine nucleotides in lung, a somewhat different story unfolds. They are significantly lower than in controls, 4, 9 and 12 hours after paraquat, whereas diquat does not produce such a drop. We thought that this would indicate a perhaps transitory incapability of paraquat-exposed lungs to resynthesize lost or destroyed nucleotides. This possibility was verified by measuring the *in vivo* incorporation of glycine into total adenine. After i.v. injection, glycine

incorporation in lung is linear over a 2-hour period. Both after paraquat and diquat, we found an initial inhibition of glycine incorporation into total adenine. However, 4 hours after diquat and 24 hours after paraquat, incorporation was much higher than in controls. We did not detect any changes in the specific activity of the glycine pool or in the size of the total adenine pool; the increased incorporation of the label might therefore reflect true increase in synthesis. But the essential conclusion is that neither paraquat nor diquat interferes, within 24 hours, with the lung's capability to step up de novo adenine synthesis.

Several experiments with oxygen also proved inconclusive. Paraquat toxicity is greatly enhanced if animals are exposed to an atmosphere of 100% oxygen (Fisher et al., 1973). If depletion of NADPH were instrumental in paraquat toxicity, then one could anticipate two things: in paraquat-injected rats kept in oxygen, NADPH would be much more oxidized than in animals kept in air. This was not quite so; 60 minutes of oxygen did not oxidize appreciably more NADPH after a dose of 40 mg/kg of paraquat than did air, and after a low dose (5 mg/kg) the NADPH/NADP ratio was practically normal. Eight hours of oxygen exposure seemed to aggravate both the effects of 5 and 40 mg/kg of paraquat. However, the effects were in no apparent relation to the clinical conditions of the animals, which were at this time practically moribund, showing grossly damaged lungs. These observations make it hard to assume a straightforward relation between NADPH oxidation and paraquat toxicity.

If our own observations presented so far do not support the view that depletion of NADPH is causally related to the toxic effect of paraquat, data obtained by Smith and Rose in England support the view (1976). These authors have evidence to show that paraquat accumulates in specific lung compartments. In our experiments, we measured NADPH concentrations in total lung and therefore know nothing about compartmentalization nor the rate at which NADPH is depleted and reformed. But we also found it necessary to reexamine another assumption underlying much work done with paraquat: is diquat really nontoxic to the lung? Recent work by Drs. Côté and Hirai showed clearly that diquat has also an effect upon lung ultrastructure. The data can be summarized as follows: overall, the morphologic changes observed 24 hours after diquat (for example, cytoplasmic edema and loss of organelles in type I alveolar cells) are quite

similar in nature and intensity of changes seen between 6 and 12 hours after paraquat. This makes the use of diquat as a "control compound" somewhat questionable.

I wanted to illustrate with these examples my first point; biochemistry should and will help to unravel mechanisms of toxicity in lung. However, there are many pitfalls and blind alleys. And I always like to recall what was said some time ago in a similar situation with regard to understanding toxic liver damage in biochemical terms: "Knowledge will advance by attrition" (Judah, 1969).

The next point I want to make is how biochemistry may help to follow the development of a toxic lesion in lung. In 1972 a paper appeared (Marino and Mitchell, 1972) describing some hitherto unrecognized effects of the antioxidant butylated hydroxytoluene (BHT) on mouse lung. The morphologic data appeared to indicate that BHT would induce cell proliferation. We verified this by measuring the *in vivo* incorporation of thymidine into total pulmonary DNA. Two days after a single intraperitoneal injection of 400 mg/kg BHT, DNA synthesis increased, reached a peak around 4-5 days and then fell off again. That DNA synthesis indeed took place was confirmed by the observation that total DNA per lung, determined chemically, increased proportionally to the dose of BHT administered (Witschi and Saheb, 1974). Measuring the amounts of protein, lipids, and RNA allowed a further conclusion: over a 5-day period, BHT not only produced a net increase in total amounts of proteins, lipids, and nucleic acids per lung, but also substantially increased the ratio of RNA/DNA. The data indicate that BHT produces, in mouse lung, cell hyperplasia and cell hypertrophy (Saheb and Witschi, 1975).

Comparatively simple biochemical methods allow one to follow changes in lung produced by toxic agents. However, we need morphology if we want to interpret the biochemical changes correctly. Proof that BHT indeed produced cell proliferation was obtained by determining, with autoradiography, the labelling index of the alveolar cells. A look at lung tissue shows that the nuclei of many cells become labelled with tritiated thymidine after BHT. A time study revealed that 1 day after BHT, the percentage of thymidine labelled cells did not exceed control values. It then rose sharply to 6% and remained at this

value to the 5th day; subsequently thymidine incorporation dropped to near normal levels by the 9th day. The activity of two enzymes involved in nucleic acid biosynthesis, thymidine kinase and uridine kinase, followed an essentially similar pattern (Adamson et al., 1976). The autoradiographic experiment thus confirmed what we had concluded from biochemistry - BHT produces cell growth. And since biochemical techniques are less time-consuming, we may use them to follow this proliferative response in lung. For example, in one series of experiments we examined whether it was possible to modify the BHT-induced lesion by several drugs. Pretreatment with phenobarbital seemed to mitigate the proliferative response. Three days after BHT the activity of pulmonary thymidine kinase was lower throughout in animals pretreated with phenobarbital. Thus, a purely biochemical technique serves as a convenient endpoint when it comes to judge the success of a treatment designed to modify cell proliferation in lung.

To understand why BHT causes cell proliferation, we needed electron microscopy. It is now established that BHT probably does not simply stimulate cells to divide, but rather causes within the first 24 hours extensive lung damage. Many type I alveolar cells become edematous and necrotic; they detach from the basement membrane. This defect is then quickly repaired by an intensive proliferation of the type II alveolar cells (Hirai et al., in preparation). Cell growth in lung after BHT is therefore simply tissue repair; BHT initially causes cell death and does not, as we thought originally, stimulate cell growth without causing damage first.

Therefore, morphologic and biochemical studies have to be done in parallel if we want maximum insight into mechanisms of toxic lung damage. Morphology gives us an indication of what is going on. However, the limitation to a detailed analysis is the time it takes to prepare and to evaluate representative samples. Biochemical techniques do not suffer from such disadvantages although the measurements only provide us with average information on changes. Moreover, the changes have to be rather substantial if we can hope to pick them up and quantitate them with reliability.

But once we find a useful biochemical marker, biochemistry allows us to quantitate what is going on. Earlier autoradiographic studies have shown that cell division is adversely affected in the lungs of animals exposed continuously to an atmosphere of 100% oxygen (Adamson et al., 1974; Evans and Hackney, 1972). We decided to verify this by injecting mice with BHT and, on subsequent days, to expose them to an atmosphere of 100% oxygen. An inhibition of *in vivo* incorporation of thymidine into DNA, a biochemical marker of cell division, could then indicate the cytotoxic effect of oxygen.

In preliminary experiments, we measured *in vivo* DNA synthesis 2, 3, and 4 days after 400 mg/kg of BHT. Sixteen or 24 hours before measuring thymidine incorporation, groups of 10 animals were put into a plexiglass chamber ventilated with 100% oxygen. An equal number of controls was kept in an identical chamber, ventilated with compressed air. It was found that exposure to oxygen for 16 or 24 hours depressed DNA synthesis. It was also noted that inhibition was greatest 2 days after BHT and least after 4 days.

We then chose the time of maximum effect, 2 days after BHT, to test dose-response relationship. Animals were exposed for 16 hours to 100% oxygen or 80%, 60%, and 40% oxygen in nitrogen. The experiment was done twice: first, as in the preliminary experiment, with animals fed ad libitum in the exposure chamber. However, oxygen makes the animals lose appetite and this alone may depress DNA synthesis (Hackney et al., 1976). A second experiment was thus run with no access to food. The data agree rather well and seem to allow establishing a threshold for 16 hours oxygen toxicity, as defined by significant inhibition of thymidine incorporation, somewhere between 60% and 40% oxygen.

The data may be used to verify Haber's rule (McFarland, 1976): for a given effect, $C_1 t_1$ should equal $C_2 t_2$. From the few data we have so far, it would seem that the relation is correct: 16 hours of 60% reduce DNA synthesis to 60%. A similar drop should be seen after 9.6 hours of 100% oxygen. Experimentally, we found that an exposure for 10 hours to 100% oxygen drops DNA synthesis to 47% of controls. The rule seems thus to be reasonably well followed, although we will need additional data before definite conclusions can be drawn.

We must realize that measurement of overall DNA synthesis is a crude endpoint if we are dealing with a mixed cell population such as in lung. We were also somewhat surprised to see that the response to oxygen appeared to be less serious 4 days after BHT than at 2 days after BHT. A time study was done on these two days with no food in the exposure chambers in order to exclude a fasting effect. Again, we confirmed that oxygen disturbs cell proliferation more at two days after BHT than it does after 4 days. Moreover, first significant changes were seen as early as 10 hours after oxygen exposure.

As a second parameter of oxygen toxicity, we measured incorporation of leucine into protein. We could confirm our observation made by measuring DNA synthesis although the overall effects were less marked; two days after BHT, oxygen had a greater effect than after 4 days.

A possible clue to this puzzling phenomenon might be given by morphology. A cytodynamic analysis showed that the initial rise in DNA synthesis following BHT injection was almost entirely accounted for by a labelling of type II alveolar cells, with peak values on days 2 and 3. This was followed by a rise in interstitial labelling on day 4, followed by a plateau. Endothelial labelling showed a late peak at 6 days only.

This leads us to the following tentative interpretation of our data on oxygen toxicity: oxygen toxicity, as measured by in vivo inhibition of DNA synthesis is more pronounced early after BHT, when most cells in division are type II epithelial cells. It could be that these cells are more susceptible to oxygen than are interstitial cells. This is undoubtedly an intriguing hypothesis and it is important to test it. Type II cells are the stem cells of the pulmonary epithelium. They are vital in repairing damage to type I cells (Witschi, 1976). Such damage may be brought about not only by inhalants but also by agents reaching the lung via the bloodstream. For example, drugs can produce type I cell damage. We should therefore be alert to the possibility that oxygen, and possibly other oxidants, might not only cause lung damage per se but also interfere with the repair process in the pulmonary epithelium.

This hypothesis could be formulated because biochemistry allowed us to collect a fair amount of quantitative data; we could never have gathered as much information with morphologic techniques alone. However, to prove or to disprove our hypothesis, we will ultimately need a detailed cytodynamic analysis of the oxygen effect. This will have to be done with established morphologic procedures. Our biochemical data will help to plan the best possible experiment. They also have given us confidence that a time-consuming morphologic study is well worthwhile.

I want to point out that most investigators studying lung damage recognize this need for a close collaboration between biochemist and morphologist. Analysis of toxic lung damage may well become one of the most challenging fields in toxicology and I anticipate that much progress will be made in the near future. I wish also to thank the people who did the morphologic work in my studies with BHT, Drs. Adamson and Bowden from Winnipeg, and Drs. Côté and Hirai from the Department of Pharmacology, University of Montreal.

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DEPOSITION AND CLEARANCE OF INHALED PARTICLES:
COMPARISON OF MAMMALIAN SPECIES*

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"Unless a new approach materializes, future efforts to study alveolar clearance will be expected to follow two paths: (a) the development of better and safer aerosols for use in man; and (b) the pursuit of basic information on alveolar clearance mechanisms. Whether the first of these is warranted without a major effort toward the second is extremely doubtful."

--Paul E. Morrow, 1973

INTRODUCTION

Deposition and clearance phenomena of inhaled particles are of fundamental importance in aerosol inhalation toxicology for several reasons. First, deposition efficiencies and clearance rates along with metabolic processes together are the determinants of tissue doses from inhaled toxins. These factors must also weigh on selection of species and extrapolation of data obtained in one species to other species. Also, particle sizes in aerosol inhalation experiments must be selected with deposition and clearance patterns in mind. Failure to do so may result in failure to dose the experimental subjects properly.

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Understanding deposition and clearance phenomena in one or more species necessitates detailed investigations into the underlying factors and mechanisms, physical, chemical and biological, that control the capture of inhaled particles within the respiratory tract and their subsequent redistribution, transformation and elimination. Among these controlling factors are:

1. Respiratory tract anatomy including airway lengths, diameters, branching angles, inclinations to gravity, radii of curvature, shapes and orientations of flow dividers, lung volumes, surface areas, etc.
2. Air flow and breathing characteristics including air velocities, Reynolds' numbers, air mixing patterns, secondary flow patterns, respiratory rate, depth, breath shape characteristics and compartmental air volumes.
3. Particle characteristics such as geometrical size, dispersion of size, mass, shape, density, surface area, electrical charge, hygroscopicity, number of particles per unit volume of air, dissolution rate in "lung" fluids, etc.
4. Physiological and morphological status including distribution, beating rate and synchrony of cilia, quantity and properties of mucus, macrophage numbers, distribution and movement and engulfment rates, status of lymphatic flow, tissue thicknesses, etc.

The above list is likely to be incomplete in that several controlling factors are probably yet to be discovered. Until a more complete theoretical understanding of deposition and clearance phenomena is secured, we must turn to the scant existing empirical data for practical information on the subject. Fortunately, sufficient data exist to make a general comparison of deposition and clearance phenomena in human, donkey, dog, rat, guinea pig, hamster and mouse.

DEPOSITION AND CLEARANCE IN THE HUMAN RESPIRATORY TRACT

Data for man were summarized in 1966 by the Task Group on Lung Dynamics of the International Commission on Radiological Protection in their publication, "Deposition and Retention Models for Internal Dosimetry of the Human Respiratory Tract"

(1966). The task group model divides the respiratory tract into three compartments, nasopharyngeal, tracheobronchial and pulmonary and describes deposition probabilities as a function of aerosol aerodynamic diameter and describes clearance rates on the basis of site of deposition and particle solubility characteristics. The task group publication is required reading for anyone involved in aerosol inhalation toxicology.

Since the human is the animal of central concern, one must ask how well the task group model has been validated by studies reported since its publication. With respect to deposition of particles a recent review by Mercer (1975) led to the following conclusions.

1. The deposition probability curves for total deposition and nasal deposition are verified for nasal breathing.
2. The curves for total deposition are verified for mouth breathing except that the data of Heyder (1975) indicate lower values for most particle sizes.
3. The task group model underestimates tracheobronchial deposition; the model used apparently did not include a sufficient number of impaction sites.
4. And therefore, pulmonary deposition is overestimated. For example the pulmonary deposition of 3 micrometer (aerodynamic diameter) particles given as about 22% should probably be about 18%.

In addition to Mercer's conclusions, it appears that the task group deposition model is not easily applied to certain types of aerosols; e.g. strongly hygroscopic ones, or highly concentrated ones (in terms of particles per cc of air) such as undiluted cigarette smoke where colligative or cloud phenomena are of importance.

With respect to clearance, the task group nose appears to clear very insoluble particles (i.e. those with slow rates of dissolution) too rapidly (Morrow, 1970). The residence half-times for very insoluble materials in the pulmonary region can be much longer than the maximum 360 days predicted by the task group model. For example, Bianco (1974) has shown the clearance half-time for tantalum in the deep lung may be greater than 850 days. As noted by the task group, deep lung persistence is

probably best predicted by the dissolution rate constant [units of grams/(cm² x time)] (Mercer, 1967) for a material in the lung environment. Unfortunately this parameter, difficult to measure *in vitro*, has not been obtained for many materials, and is not always related to the equilibrium water solubility constant. Data and discussions on this topic are available (Morrow, 1964, 1968m 1972; Mercer, 1967; Kenoyer, 1975; Kamapilly, 1973).

For the purposes of this paper, the task group lung model promotes an adequate description of deposition and clearance phenomena in humans.

ANIMAL DEPOSITION DATA

Before deposition data in animals can be utilized in making interspecies comparisons the study must have been relatively complete. For example, the aerodynamic size of the aerosol must be given and the absolute or relative deposition in anatomical regions measured. Few studies conform to these standards, so an abundance of data are not available. However, sufficient information exists to warrant a first-order comparison of selected species.

Experimental studies at New York University by Albert, Lippman and others indicate that spontaneously breathing donkeys have a higher fractional tracheobronchial deposition efficiency than humans for monodisperse particles near 5 micrometers aerodynamic diameter (Albert et al., 1974).

Values for deposition of radioactive aerosols in dogs have been reported by Cuddihy et al. (1973) at the Lovelace Foundation's Inhalation Toxicology Research Institute. Nose-only exposures using anesthetized dogs were to polydisperse aerosols ranging from 0.4 to 6.6 micrometers aerodynamic diameter. After deposition, dogs were periodically scanned longitudinally, and integration under peaks gave amounts of aerosol in various anatomical regions. The experimentors concluded that total deposition, though slightly less than that seen in humans, was in close agreement with the task group values. Only nasal deposition was significantly less than that in man. The slower breathing rate (mean of 12 per minute, about half of that normal for awake dogs) could, however, explain this observation. Heyder (1975), for example, has shown nasal efficiencies in humans to be lower at lower air flow rates.

With respect to smaller animals, work from the Inhalation Toxicology Research Institute (Raabe et al., 1974) involved monodisperse radiolabeled fused clay particles in five size ranges from 0.05 to 3.0 micrometers aerodynamic diameter.

Lightly anesthetized rats and golden Syrian hamsters inhaled the aerosols (nose only) for 20 minutes. Breathing rates per minute, as determined by body plethysmographs, were 68 for rats and 59 for hamsters. Half of the animals were killed and dissected 24 hours postexposure. Organ analysis included skull, gastrointestinal tract, larynx, trachea, and each lobe of the lungs. No significant differences in deposition were seen between the two species but total deposition efficiencies were less than those for humans. Again, the nose was less efficient than that of humans.

Data published by the late Glen Fairchild and others (1975) of the Environmental Protection Agency on the deposition of 2.6 micrometer diameter radiolabeled bacteria in guinea pigs are in very close agreement with Raabe's deposition data for rats.

Deposition data in rats for 2.5 micrometer (aerodynamic median diameter) particles published by Johnson and Ziemer (1976) are slightly higher than those of Raabe et al. (1974) but still in substantial agreement.

In general, deposition efficiencies in various sections of the respiratory tract appear remarkably similar in humans and non-human mammals. When differences in lung, body size, and tidal volume are taken into account, one concludes that smaller animals receive a greater dose per unit lung or body weight. For particles near 1 micrometer in diameter, the rat can be expected to get a dose roughly 5-10 times that of man and the dog about 3 times that of man on a per gram organ basis. Such extrapolations of dose in animal inhalation studies are hazardous since actual deposition may differ markedly from the predicted by animals avoiding exposure (nuzzling against one another, burying noses in fur or cage corners) or by unexpected behavior of the aerosol (losses to electrically charged fur, hygroscopicity, etc.).

DEPOSITION MODELING IN ANIMALS

Mathematical prediction of aerosol deposition in animals other than humans for first principles requires detailed quantitative knowledge of respiratory tract anatomy and of air flow and mixing in the lung. To date, such information is not available. Yeh et al. (1976) have described the elements required for

such deposition calculations and reviewed the models used for humans. A similar approach to predictive animal models involves extrapolation of human models to other species. Klimet of the Military Institute of Hygiene, Epidemiology and Microbiology, Prague, published "Similarity and Dimensional Analysis, Evaluation of Aerosol Deposition in the Lungs of Laboratory Animals and Man" in 1973. He listed the parameters that influence particle deposition and concluded that if deposition is known in any one species it can be extended to any other from a consideration of airway lengths, diameters, tidal volume, respiratory rate, body weight and volume of airway zones. This concept, though not yet validated, is certainly an aid to those interested in generating data on airway geometry.

Stauffer of the Institute of Theoretical Physics, West Germany, described a scaling theory for aerosol deposition in animals (1975). Assuming similarity of all species with respect to number of bronchi, number of alveoli, ratio of tracheal length to alveolar diameter, generation-by-generation flow patterns and ratio of tidal volume to total lung volume, he predicted no significant differences in deposition between species. Again, though the assumptions are not strictly valid, the approach is a valuable, perhaps even necessary, one toward developing sound predictive models. Through such modeling attempts important gaps in knowledge in basic areas are brought to light.

PARTICLE CLEARANCE

The data, with respect to clearance of deposited inhaled particles, are much less clear than those for deposition. Measurements of clearance rates are strongly influenced by the method of measurement. Mechanisms for clearance, mucociliary transport, macrophage action, dissolution in lung fluids, etc., however, appear to be quite similar to most mammals. Therefore, one expects the same type of general similarity in clearance phenomena that is seen in deposition. This expectation does not appear to be substantiated with respect to early clearance, however. Robert Thomas of the Inhalation Toxicology Research Institute developed models for clearance of insoluble particles from the respiratory tract of dog, rat, and mouse (1972) (Figure 1). Similar data have been described, though not published, by Cuddihy (1973), also of the Inhalation Toxicology Research Institute, for dog, rat and hamster. At least for these animals, wide inter-species differences appear to exist in short-term clearance rates

for particles with similar properties, deposited under similar conditions. The rodents appear to have a relatively sustained rapid clearance phase at times after the disappearance of rapid clearance in the dog.

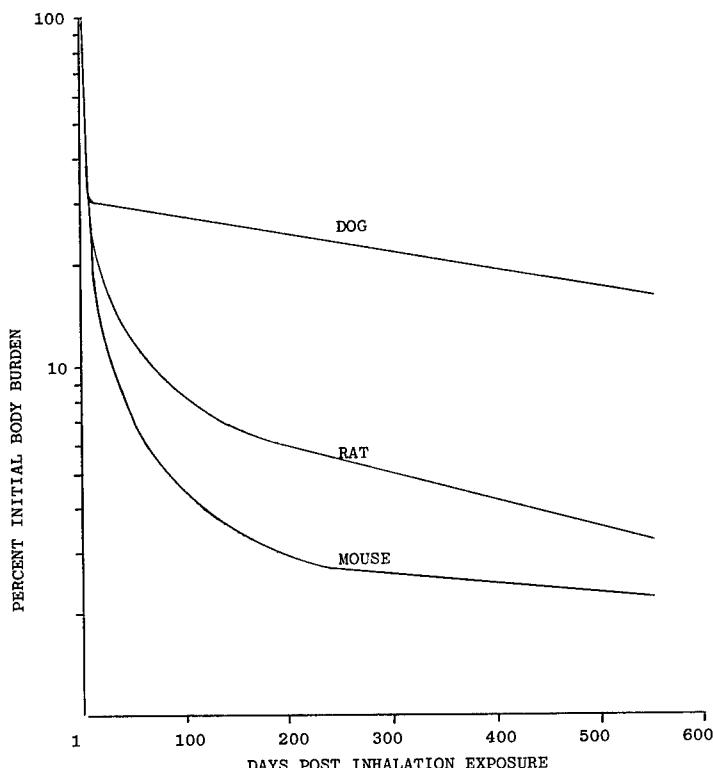


Figure 1. Theoretical clearance curves for dog, rat, and mouse lung determined by studies with relatively insoluble inhaled particles. After R. G. Thomas, 1972.

Similarly, Albert et al., of New York University, describe significant differences in early clearance between donkeys and humans (1971). Early clearance in donkeys was described as single exponential in character while that in humans was reported to be two-phase in most humans.

With respect to late clearance, presumably clearance of particles deposited distal to the ciliated airways, general similarity between species has been seen by several investigators. This finding lends weight to the hypothesis that deep lung clearance rate is dominated by particle dissolution-rate properties. A few examples will suffice to illustrate this point.

Skolil of San Diego State University found a clearance half-time of 15 days for silver inhaled by 50 persons in a nuclear reactor accident (1961). Studies of the clearance rates of inhaled silver fume particles in rats (Phalen, 1966) and in dogs (Phalen, 1973) indicated remarkably similar clearance half-times of 13 days in both species. Hursh and Mercer (1970) found a clearance half-time in man of 11.9 hours for inhaled sub-tenth micrometer lead particles. Subsequently, Bianco (1973) found a clearance half-time for small lead aerosols, inhaled by dogs, of 11.9 hours. In both of these studies clearance rate determinations were made by external radiation detection measurements and careful corrections were made for interfering counts from radioactive lead dissolved in blood circulating in the thorax. Also, Bianco (1974) found a clearance half-time for inhaled tantalum in dogs of about 860 days and compared this to a value quoted from Sill (1969) of about 1000 days for tantalum dioxide in humans. Where data exist for comparing species, deep lung clearance rates appear to be similar for a variety of species; however, the number of materials examined in multiple species under similar conditions is scant.

EFFECTS OF TOXIC AGENTS ON CLEARANCE

For cigarette smoke and some other agents, the effects of toxic agents on clearance of particles appear to be remarkably similar for various mammals. Again, the work of Albert, Lippman and others at New York University can be used to compare humans and donkeys (Albert et al., 1974, 1969, 1975; Lippman et al., 1973). Though the effects are strongly dependent on dose and temporal factors, humans and donkeys exposed to cigarette smoke both exhibit periods of stasis in early particle clearance. This result has been seen in man by Sanchis et al. (1961), by Phalen et al. (1975) in rats, and by Holma in rabbits (1969). The effect can presumably be explained by the ciliastatic effects of cigarette smoke components. Species similarities with respect to the effects of SO₂, cold, and various drugs on particle clearance have been reported (Ferin and Leach, 1973; Lippman et al., 1973; Bohning, 1975; Curtis, 1976; Spiegelman et al., 1968).

Though one cannot assume a priori that related agents will always effect clearance phenomena similarly in all mammals, the data do support the use of animals in toxicology studies in which disturbances in clearance are examined.

CONCLUSIONS

Though perhaps bold, based upon this modest review, several general conclusions seem possible.

1. The total deposition efficiency of aerosols in the respiratory tract is similar under similar conditions for mammalians of similar body size.

2. Smaller animals generally deposit less aerosol per unit time during exposure but more aerosol per unit body weight per unit time during exposure.
3. The distribution of deposited aerosol within the respiratory tract is probably similar for most mammals, with the human nose probably receiving a greater fraction of the total than the noses of dogs or small rodents.
4. Characteristics of early clearance are apparently highly species-specific.
5. Clearance rates from the deep lung are similar for several species, especially when particle dissolution rate is a dominant factor.
6. The effects of various agents on clearance are often similar for many materials when differing dose factors in differing species are taken into account.

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EFFECTS OF METALS ON PULMONARY DEFENSE MECHANISMS
AGAINST INFECTIOUS DISEASE

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INTRODUCTION

The respiratory system under normal conditions has the ability to maintain sterility of the deep lung even when large numbers of microbes are deposited under laboratory conditions. A number of mechanisms have been shown to be responsible for this defense of the lung, namely, mechanical clearance via the mucociliary escalator, biological clearance mediated through the alveolar macrophage (AM), and associated cellular and humoral immunological events.

The adverse effects of numerous gaseous pollutants on these pulmonary defense mechanisms against infectious disease have long been recognized and have been reviewed by Coffin and Gardner (1972) and Gardner and Graham (1977). Recently, researchers have begun to investigate the propensity of trace metals for causing similar effects. This paper, while including the relevant work of other researchers, will focus on the recent and ongoing studies in our laboratory on the toxicity of metals for these host defense mechanisms.

This report has been reviewed by the Health Effects Research Laboratory, U.S. Environmental Protection Agency, and approved for publication. Mention of trade names or commercial products does not constitute endorsement or recommendation for use.

TOTAL ASSAULT ON DEFENSE MECHANISMS

One of the most sensitive tools for research in this area is termed the "infectivity model." This model system measures the results of the interaction of chemical agents with aerosols of viable Streptococcus pyogenes. Numerous parameters of host response can be measured, such as mortality, in situ bacterial growth kinetics, pathology, etc. Using this system, the effects of the chloride and sulfate salts of Cd and Ni and the oxide and chloride salt of Mn have been investigated by Gardner et al. (1976) and Adkins and Gardner (1976). In these studies, mice (CD-1, female, 20-25g, Charles River) were exposed to respirable aerosols of the appropriate metal or to filtered air for 2 hours. Immediately, or 24 hours postexposure, these animals received an aerosol of Streptococci. Figure 1 presents the combined data from these studies. The response to these metallic compounds differed. Concentrations of $\text{CdCl}_2 \geq 90 \mu\text{g Cd/m}^3$ and $4750 \mu\text{g Mn/m}^3$ as Mn_3O_4 significantly increased mortality ($p < .05$) when mice were exposed to the bacteria immediately after exposure. No statistically significant effects were observed with the MnCl_2 concentrations tested. Also, no statistically significant mortality increase was evident with Ni unless the infectious challenge occurred 24 hours postexposure to $500 \mu\text{g Ni/m}^3$ as NiCl_2 . The influence of the anion was also interesting. While the effect of CdCl_2 was little different from CdSO_4 , the chloride form of Ni appeared to be more damaging than the corresponding sulfate. The oxide of Mn seemed to be more deleterious than the chloride form.

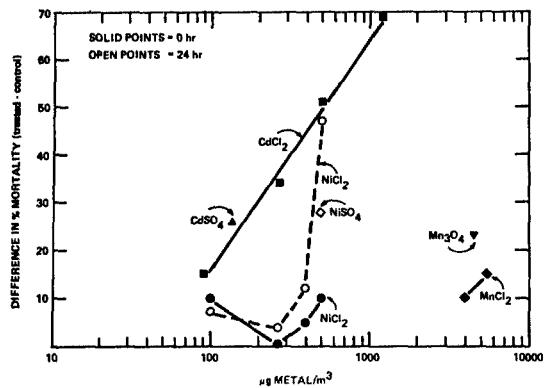


Figure 1. Percent enhancement in mortality (difference from control) on day 15 following a 2-hour exposure to aerosols of the indicated metals. The mice were challenged with viable Streptococci at 0 time (solid points) or at 24 hours (open points) following cessation of pollutant exposure. All points above 20% are significantly different from controls ($p < .05$).

The only exception is CdCl_2 at $90 \mu\text{g Cd/m}^3$, which was also statistically significant. (From Gardner et al., 1976; Adkins and Gardner, 1976).

In order to compare directly the toxicity of the pollutants used in these experiments, $\mu\text{g metal/m}^3$ have been converted to $\mu\text{g atoms metal/m}^3$. In this way it is possible to compare the effect of an exposure to a certain number of atoms of one metal to the same number of atoms of another metal. Therefore, for comparisons in the text, both forms of metal concentration will be used. However, since $\mu\text{g metal/m}^3$ is the more conventional expression, this form will be used on tables and graphs. When the data on Figure 1 are examined as $\mu\text{g atoms metal/m}^3$, the relative relationships remain the same. For example, the 500 $\mu\text{g Cd/m}^3$ (4.44 $\mu\text{g atoms Cd/m}^3$) exposure is similar to the 275 $\mu\text{g Ni/m}^3$ (4.68 $\mu\text{g atoms Ni/m}^3$) treatment. Conversion of all the numbers has the net effect of shifting the Ni points to the right, thereby further illustrating the greater toxicity of Cd.

Maigetter et al. (1976) have reported on the effects of a MnO_2 aerosol using a similar infectivity model. In these experiments, mice were exposed to 109 mg Mn/m^3 (as MnO_2) 3 hr/day for various lengths of time prior to a challenge with airborne Klebsiella pneumoniae or after an aerosol exposure to influenza virus. Mice exposed for 3 or 4 days and challenged with bacteria immediately after metal exposure had significantly ($p = .08$) higher mortality indices as compared to bacteria exposed controls. When influenza was used as the infectious agent 24 hours before a 3 hour exposure to MnO_2 began, there was a significant ($p < .05$) enhancement of mortality.

Using the infectivity model, prior studies by Coffin and Gardner (1972) and Gardner et al. (1976) have indicated that death is preceded by bacterial invasion of the blood which occurs following increased bacterial growth in the lungs. Furthermore, the mortality seen is thought to represent the sum of an assault on a number of defensive systems - mucociliary clearance, the AM, and the immune system.

MECHANICAL CLEARANCE

As a number of investigators have reported, the mucociliary escalator is responsible for clearing approximately 10-25% of an inhaled bacterial load (Goldstein et al., 1974; Kim et al., 1976; Fairchild and Stultz, 1976). Therefore, if this system were completely destroyed, other deep lung clearance mechanisms could still proceed, but at a decreased level.

In order to investigate the effects of pollutants on ciliary activity, *in vivo* and *in vitro* models were used by Adalis et al. (1976a, 1976b). Hamsters were exposed to respirable sized aerosols of NiCl_2 or CdCl_2 for 2 hours. Immediately, and at various days postexposure, the trachea were excised and cut into rings. The rings were cultured and ciliary beating frequency determined by synchronizing the beating with a calibrated stroboscopic microscope attachment. In the *in vitro* system, tracheal rings were exposed to the metals in tissue culture. Measurements were made over a 3 day period.

Figure 2 shows the results of a 2 hour exposure of hamsters to various concentrations of CdCl_2 or NiCl_2 for either 1 or 2 days. Ciliary beating frequency was determined immediately following cessation of exposure. The hamsters exposed to $275 \mu\text{g Ni/m}^3$ ($4.68 \mu\text{g atoms Ni/m}^3$) received much more metal than those animals breathing $50 \mu\text{g Cd/m}^3$ ($0.44 \mu\text{g atoms Cd/m}^3$), but the depression in ciliary beating was quite similar. Therefore on the basis of $\mu\text{g metal/m}^3$ or $\mu\text{g atom metal/m}^3$, Cd was more toxic than Ni. Twenty-four to 72 hour *in vitro* treatments to $11 \mu\text{M NiCl}_2$ or $12 \mu\text{M CdCl}_2$ also significantly ($p < .05$) depressed ciliary activity. Following the 24 hour incubation, Cd^{2+} was more toxic than Ni^{2+} .

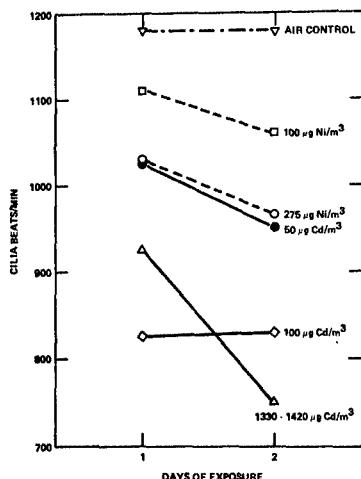


Figure 2. Beats/minute of cilia from the trachea of hamsters exposed to metal chlorides or filtered air for 2 hr/day for either 1 day or 2 days. All treatment means are significantly different from controls ($p < .05$) (From Adalis et al., 1976a, 1976b).

When animals were allowed to recover for 24 hours following the in vivo exposure to $100 \mu\text{g Ni/m}^3$ (2 hr/day X 2 days), significant depression in ciliary activity was still evident. Recovery had begun within 48 hours and was complete by 72 hours. Seven days following the end of a 2 day exposure to $275 \mu\text{g Ni/m}^3$ (2 hr/day), ciliary beating frequency had not returned to control values ($p < .05$). Animals that received this pollutant for only one day exhibited normal ciliary activity at 7 days postexposure. Beating frequency of cilia from hamsters exposed to 50, 100, and $500 \mu\text{g Cd/m}^3$ was also significantly depressed during a 48 hour recovery period.

BIOLOGICAL CLEARANCE

BACTERIAL CLEARANCE

The ability of a 2 hour exposure to CdCl_2 (Gardner et al., 1976) or NiCl_2 (Adkins and Gardner, 1976) to depress in situ clearance of viable Streptococci administered by aerosol is depicted on Figure 3. CdCl_2 exposed animals received the bacterial challenge immediately following pollutant exposure, while the mice exposed to NiCl_2 did not receive the Streptococcal aerosol until 24 hours post Ni treatment. At the indicated time periods, the lungs of pollutant and clean air exposed mice were homogenized and viable bacterial counts determined. The microorganisms in the lungs of exposed animals grew, while those deposited in animals exposed to filtered room air exhibited a net reduction. The exposure to $500 \mu\text{g Ni/m}^3$ (8.15 $\mu\text{g atoms Ni/m}^3$) was, on the basis of $\mu\text{g atoms metal/m}^3$, almost 3 fold higher than the aerosol of $325 \mu\text{g Cd/m}^3$ (2.89 $\mu\text{g atoms Cd/m}^3$). This difference in concentration was not reflected in the difference between the effect of the metals on bacterial growth. Therefore while a directly determined ranking of toxicity cannot be made, the data do suggest that Cd was more potent than Ni in promoting a net increase in bacteria within the lung.

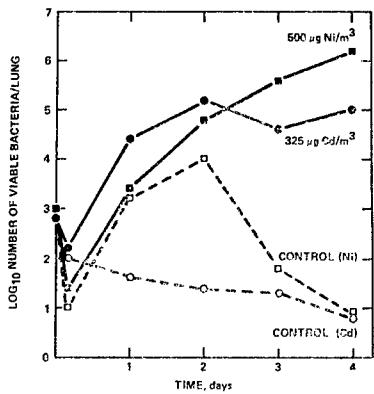


Figure 3. Log number of viable Streptococci/lung at various times following cessation of bacterial aerosol. Mice were exposed for 2 hours to aerosols of either metal chloride aerosol or filtered air immediately prior to bacterial challenge in the case of CdCl_2 or 24 hours prior in the case of NiCl_2 (From Gardner et al., 1976; Adkins and Gardner, 1976).

Within the first week after the microbial aerosol, Streptococci began to appear in the blood (Gardner et al., 1976; Adkins and Gardner, 1976). Greater numbers of mice in the CdCl₂ and NiCl₂ exposure groups had bacterial contamination of their blood, as compared to corresponding control animals. A more extensive analysis of the data from CdCl₂ and air exposed animals showed a strong positive correlation between bacterial invasion of the blood and the mortality observed in the infectivity model.

ALVEOLAR MACROPHAGE

Investigators have attributed deep lung sterility to the proper functioning of the alveolar macrophage (Goldstein et al., 1974; Kim et al., 1976). However, the previously mentioned metals as well as other pollutants (Gardner and Graham, 1977) are capable of adversely affecting this function.

The ability of AM isolated from rats exposed for 2 hours to 500 µg Ni/m³ (as NiCl₂) to phagocytize 1 µm latex spheres was determined (Adkins and Gardner, 1976). The cells harvested immediately after pollutant treatment exhibited phagocytic activity similar to cells from clean air exposed animals. However, AM lavaged 24 hours postexposure had a 19% reduction in phagocytic activity as compared to control. At this time period, a 6% reduction in ATP was observed. Interestingly, decreases in ATP, phagocytosis, and the enhancement of mortality in the infectivity model were only observed 24 hours following Ni exposure.

The total number of cells and the differential composition of cells lavaged from the lungs can also be altered by Ni or Cd treatment. Bingham et al. (1972) exposed rats for 12 hr/day, 6 days/week for 2 weeks to 109 µg Ni/m³ (as NiCl₂) or 120 µg Ni/m³ (as NiO). Rats breathing the NiCl₂ had an increase in total number of recoverable cells (146% of control), while animals exposed to the NiO had an even greater increase (280% of control). Adkins and Gardner (1976) exposed mice and rats to 500 µg Ni/m³ (as NiCl₂) for 2 hours and found no changes in differential counts or viability of AM. However, they did find a reduction in the total number of cells recoverable from mice (62% of control) and rats (87% of control). This apparent discrepancy with the Bingham et al. (1972) study could be due to the difference in the exposure regimen.

When studying the effects of 2 hours of exposure to CdCl₂ at 500 and 1500 µg Cd/m³ on rats, Gardner et al. (1976) measured similar parameters. Immediately and at 24 hours following exposure, there was a significant reduction ($p < .05$) in the viability of AM of rats exposed to the higher concentration only. Several significant differences were found when lavaged cells from exposed animals were compared to cells from clean air exposed rats. Immediately after the exposure to 1500 µg Cd/m³, there was a reduction ($p < .05$) in the number of AM/lung (from 5.56×10^6 to 3.7×10^6), fewer total cells were recoverable, and there was an increase in polymorphonuclear leukocytes. The significant ($p < .05$) increase in total cells 24 hours post-exposure to 1500 µg Cd/m³ (from 5.54×10^6 cells/lung to 19.26×10^6 cells/lung) was primarily due to an influx ($p < .05$) of polymorphonuclear leukocytes (from 0.11×10^6 /lung to 13.06×10^6 /lung) even though there was also an increase ($p < .05$) in lymphocytes (from 0.08×10^6 /lung to 0.83×10^6 /lung). The number of AM was not different from control values 24 hours postexposure to either Cd concentration. Such changes in pulmonary cell populations could indicate inflammatory responses and possible alteration in host defense mechanisms.

The subsequent information concerning the effect of metals on AM is derived from in vitro studies. In the first series of experiments described, normal rabbit AM were allowed to attach to tissue culture bottles prior to exposure to a 0.2 µm filtrate of metal solution. Following a 20 hour incubation, the AM were harvested with trypsin and a number of parameters investigated.

The results of exposure to various concentrations of NH₄VO₃ and the chlorides of Cd²⁺, Ni²⁺, Mn²⁺, and Cr³⁺ can be described in terms of the viability index (Waters et al., 1975a). This index is determined by multiplying the % viability of AM in the test culture times the ratio of the number of cells in the test culture to the number of cells in controls. This is an expression of intact viable cells remaining after exposure. The results are expressed as EC₅₀ (mM concentrations of metallic ions causing a 50% reduction) (Table 1). Cadmium chloride and ammonium vanadate were most potent in reducing the viability index and specific activity of acid phosphatase. The other chlorides of Ni²⁺, Mn²⁺, and Cr³⁺ were less cytotoxic than Cd²⁺ and VO₃⁻.

TABLE 1. EC₅₀ (mM)^a OF METALLIC IONS CAUSING
DECREASE IN VIABILITY INDEX AND ENZYME ACTIVITY
(FROM WATERS ET AL., 1975a)

<u>Ion^b</u>	<u>Viability Index^c</u>	<u>Acid Phosphatase Specific Activity</u>
Cd ²⁺	0.08	0.20
VO ₃ ⁻	0.10	0.09
Ni ²⁺	3.78	3.80
Mn ²⁺	4.67	5.31
Cr ³⁺	5.06	4.44

^amM concentration of metals causing a 50% reduction in activity following a 20 hour in vitro exposure in tissue culture bottles.

^bMetallic chlorides were used with the exception of VO₃⁻, in which case NH₄VO₃ was used.

^cViability Index = Viability (%) X $\frac{\text{Total intact cells in test culture}}{\text{Total intact cells in controls}}$.

Further studies of viable, metal-exposed cells indicated that phagocytosis of 1 μ m latex spheres was also reduced at the following molar concentrations of metal: 2.2×10^{-5} Cd²⁺, 3.1×10^{-3} Mn²⁺ and 5.1×10^{-4} Ni²⁺ (Graham et al., 1975a). Other relationships can also be derived from this investigation. Vanadate at 6.0×10^{-5} M caused approximately a 25% reduction in total cell numbers and a 10% reduction in viability, but had no effect on phagocytosis. Ni²⁺ at concentrations of 5.1×10^{-4} M, 7.7×10^{-4} M and 1.1×10^{-3} M, had substantial effects on phagocytosis, but had no effects on total cells remaining and minimal effects on % viability. Reductions in the phagocytic index ranged from 50 to 90%. In this system, Ni²⁺ was more toxic than Cr³⁺ or Mn²⁺. In another investigation, using multichambered Lab-Tek slides, Waters et al. (1976b, 1976c) determined the EC₅₀ for depression in phagocytic activity and ATP/mg protein following a 20 hour in vitro intubation with the chlorides of Hg²⁺, Cd²⁺, Zn²⁺, Pt⁴⁺, Cu²⁺, and Ni²⁺ (Table 2). The EC₅₀ of Ni²⁺ was almost 12 times higher than that of Cd²⁺ for ATP/mg protein. Cd²⁺ was also more toxic to phagocytic functioning.

TABLE 2. EC₅₀ (mM)^a OF METALLIC CHLORIDES CAUSING DECREASED ATP AND PHAGOCYTIC ACTIVITY (FROM WATERS ET AL., 1975b, 1975c)

<u>Metal^a</u>	<u>ATP^b</u>	<u>Phagocytic Activity^c</u>
Hg ²⁺	.04	.04
Cd ²⁺	.08	.07
Zn ²⁺	.18	.14
Pt ⁴⁺	.25	.21
Cu ²⁺	.37	.24
Ni ²⁺	.95	.33

^amM concentration of metals causing a 50% reduction in activity following a 20 hour in vitro exposure in multichamber Lab-Tek slides.

^bATP was measured as ATP/mg protein and calculations were based on % of control.

^cPercent of cells phagocytizing latex spheres was calculated as % of control.

Recent studies by Hadley et al. (1976) utilized the inhibition of antibody mediated rosette formation as an index of the effect of CdCl₂ and NiCl₂ on the Fc receptor of the AM. Rosette formation by AM was determined following a 30 minute incubation with various concentrations of the metals. Both metals inhibited rosette formation in a dose dependent manner. For example, Figure 4 shows the effect of increasing concentrations of Cd on the inhibition of antibody-mediated rosette formation. Significant inhibition of rosette formation was observed with 2.2 X 10⁻⁵ M CdCl₂. Nickel produced significant depression only at higher concentrations (1 X 10⁻⁴ M). These data suggest that the metals may directly interfere with membrane receptors of the AM.

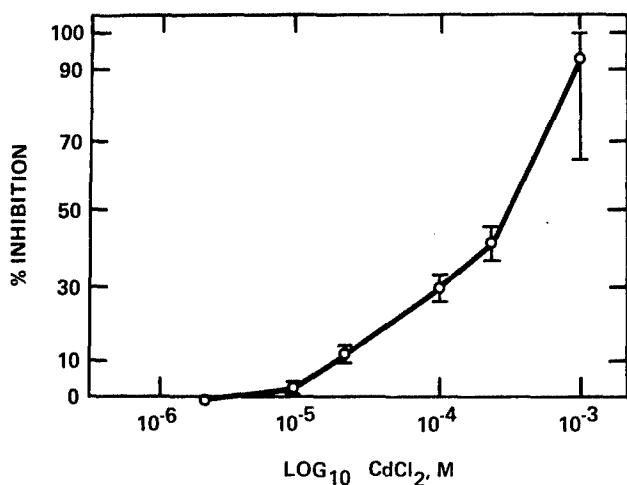


Figure 4. Percent inhibition of rosette formation by AM following a 30 minute treatment to various concentrations of CdCl_2 . Data points are mean \pm standard error (From Hadley et al., 1976).

AM have also been exposed for 20 hours to unfiltered mixtures of vanadium oxides (Waters et al., 1974). The EC_{50} for the depression in viability was found to be 0.26 mM V as V_2O_5 , 0.49 mM V as V_2O_3 , and 0.65 mM V as VO_2 . The ranking of the EC_{50} follows the relative solubility of these compounds as evidenced by further experiments which indicated that the cytotoxicity was related to the soluble or ultrafine components ($<0.2\mu\text{m}$) of the metal oxide mixture.

Since metals of environmental concern exist as complexes with different particle sizes, the influence of particle size on toxicity is an important consideration. The research of Aranyi et al. (1977) was directed to this point. Using an in vitro system, rabbit AM were exposed for 21 hours to fly ash particles coated with PbO , NiO , or MnO_2 in particle sizes of <2 , 2-5, and 5-8 μm . The percentage of metal adsorbed on the fly ash particles was similar. Since the AM were exposed to concentrations of particles, the extracellular milieu contained constant amounts of metal, irrespective of particle size. As particle size decreased, more AM phagocytized the particles. It was determined that the decreases in AM viability, total protein, and LDH activity were statistically associated with decreasing particle size. This effect was due to the metal coated particles, not to solubilization of the metals in the media. Therefore the smaller the particle, the more toxic the response. Examination of EC_{75} values for viability showed that PbO coated fly ash was more toxic for each of the size ranges studied. There was little difference between NiO and MnO_2 . This research demonstrates that in inhalation toxicology, it is important to consider the size of the particle delivered to the AM as well as the size range that will permit deep lung penetration.

IMMUNE SYSTEM

In view of the propensity of metals for enhancing bacterial pathogenesis, the study of these pollutants was expanded to include the humoral immune system due to its importance in host defense against many infectious agents. In these investigations (Graham et al., 1975b, 1976), mice were exposed to pollutants and immediately immunized intraperitoneally with sheep red blood cells. Four days later the number of antibody producing spleen cells was determined using the Jerne hemolytic plaque technique.

In the first series of experiments (Graham et al., 1976), animals were exposed to aerosols of NiCl_2 or CdCl_2 for 2 hours. Table 3 illustrates the results. NiCl_2 , at concentrations $\geq 275 \mu\text{g Ni/m}^3$, caused a statistically significant ($p < .05$) depression in the number of antibody producing spleen cells/ 10^6 cells. CdCl_2 only caused a significant suppression at $190 \mu\text{g Cd/m}^3$. If the data are compared on the basis of $\mu\text{g atoms metal/m}^3$, the concentration of $110 \mu\text{g Ni/m}^3$ ($1.87 \mu\text{g atoms Ni/m}^3$) is similar to $190 \mu\text{g Cd/m}^3$ ($1.69 \mu\text{g atoms Cd/m}^3$). This level of Cd caused a greater depression in the number of plaque producing cells ($p < .0001$) than did Ni exposure.

TABLE 3. INFLUENCE OF METAL AEROSOLS ON NUMBER OF
ANTIBODY PRODUCING SPLEEN CELLS
(FROM GRAHAM ET AL., 1976)

<u>Metal</u> <u>($\mu\text{g Metal/m}^3$)</u>	<u>Plaques/10^6 Cells</u> <u>(Control - Metal Treated)</u>
110 Cd	-95
190 Cd	280*
110 Ni	80
275 Ni	110*
380 Ni	165*
490 Ni	210*

*Significantly different from control ($p < .05$).

In another set of experiments (Graham et al., 1976) mice received NiCl_2 , NiSO_4 , NiO , CdCl_2 or CrCl_3 via intramuscular (IM) injection. The results for NiCl_2 , illustrated in Figure 5, show a significant ($p < .05$) negative linear dose response relationship. The 9.26 and 12.34 $\mu\text{g Ni/g}$ body weight treatment produced a statistically significant ($p < .05$) reduction in \log_{10} plaques/ 10^6 cells. No linear dose response relationship was found with the other metal treatments, although NiSO_4 at concentrations of 6.17, 9.26 and 12.34 $\mu\text{g Ni/g}$ body weight did cause a significant immunosuppression when compared to treatments of 3.90 $\mu\text{g Ni/g}$ or the diluent injected control group. Nickel oxide at concentrations between 3.90 to 12.34 $\mu\text{g Ni/g}$ did not depress plaque formation. From these data, one would infer that NiCl_2 and NiSO_4 are more toxic than NiO .

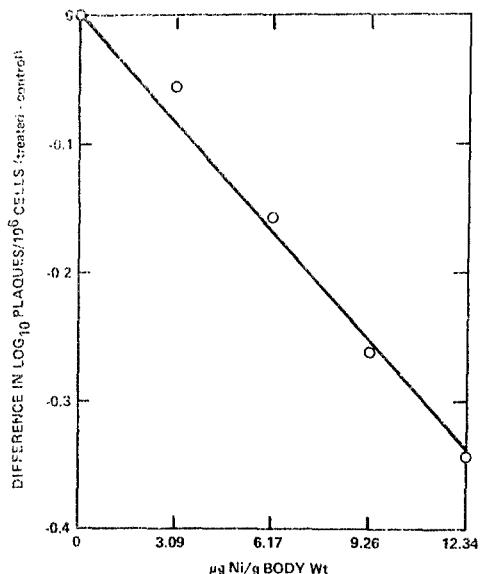


Figure 5. Difference in \log_{10} plaques/ 10^6 spleen cells (treated - control) on day 5 of mice immunized with sheep red blood cells and treated with IM injections of NiCl_2 on day 1. There was a significant linear dose response regression ($p < .05$) (From Graham et al., 1976).

Although CdCl_2 and CrCl_3 caused a slight reduction in the \log_{10} plaques/ 10^6 cells at the highest concentrations tested, there was no statistically significant difference following treatments of 1.4-12 $\mu\text{g metal/g}$ body weight. Considering the toxicity of Cd for other systems described in this paper, this lack of immunosuppression is surprising in light of the fact that the highest concentration (11.81 $\mu\text{g Cd/g}$) resulted in 17% mortality. Ni concentrations tested in this study did not affect survival. Without additional information, it is not possible to speculate on the mechanisms.

Some interesting observations can be made when the data from aerosol or IM treatment with NiCl_2 are compared. Using literature values for respiratory parameters for mice, it was calculated that the lowest effective aerosol exposure ($275 \mu\text{g Ni/m}^3$) delivered a maximum of approximately $1.94 \mu\text{g Ni}/\text{mouse}$. The lowest effective IM dose delivered an average of $208 \mu\text{g Ni}/\text{mouse}$. This represents an approximate 100 fold difference in the effective concentration. The greater potency of the aerosol route might reflect Ni clearance. Table 4 shows the clearance of Ni from the spleen following aerosol exposure and indicates that a significant amount of the metal is present 24 hours postexposure. When $6.17 \mu\text{g Ni/g}$ were injected IM, no Ni was detectable over this same time period.

TABLE 4. CLEARANCE OF NiCl_2 FROM THE SPLEEN OF MICE EXPOSED TO $625 \mu\text{g Ni/m}^3$ FOR 2 HOURS (FROM GRAHAM ET AL., 1976)

<u>Days Postexposure</u>	<u>$\mu\text{g Ni/g Dry Weight } (\bar{X} + \text{SE})$</u>
0	$1.05 \pm .086$
1	$0.86 \pm .045$
2	$0.78 \pm .065$
3	$1.35 \pm .190$
4	$1.29 \pm .373$

Often in making predictive judgements on pollutant toxicity, the concentrations in air and water are considered for their contribution to total body dose. In many cases, the air contribution is judged relatively insignificant when compared to the levels resulting from water or food. However, the results from these experiments seem to indicate that route of exposure could be as, or more important than absolute concentration in terms of resultant toxicity. Therefore such predictions should not ignore a relatively small aerosol dose unless there is experimental evidence to support such a contention.

SUMMARY

A number of metals are capable of reducing the effectiveness of host defense mechanisms. Most of the metals used in these *in vivo* investigations were used in concentrations

below the Threshold Limit Value (TLV) (American Conference of Governmental Industrial Hygienists, 1971). The TLV for these metals is as follows: 200 Cd/m³, 100 µg Ni/m³, and 5000 µg Mn/m³. In the infectivity model, Cd was more toxic than Ni. The various components of the model were affected similarly. Ciliary activity and pulmonary bacterial clearance were also more affected by Cd than by Ni.

The studies using in vitro systems with AM also show that Cd was more toxic than Ni, with EC₅₀ differences of 47, 19, 12, and 5 fold for the viability index, specific activity of acid phosphatase, ATP/mg protein, and phagocytic activity, respectively. Since the AM is primarily responsible for bacterial clearance, these effects could aid in explaining the enhancement of respiratory infection found in animal models.

Studies designed to examine the systemic humoral immune system following aerosol exposures show that Cd was more immunosuppressive than Ni on a µg atom metal/m³ basis. Concentrations of Cd aerosols which enhanced mortality in the infectivity model and decreased ciliary activity only had a slight effect on antibody production by splenic lymphocytes, but the lower (275 µg Ni/m³) NiCl₂ aerosol exposure which produced immunosuppression did not increase mortality in the infectivity model. These results are not inconsistent with the mechanisms involved in the infectivity model. Most major events associated with the infectivity model, i.e. bacterial growth, invasion of the blood, and increased mortality, begin 4-5 days following bacterial challenge. A primary immune response would just be beginning at this time (Kaltreider et al., 1974), and thus for the immune system to be highly effective in the infectivity model, a localized pulmonary primary immune response or an already existing nonspecific system would be needed. The immunological model described in this report is a systemic one. Since the best immunological protection of the lung occurs when the route of immunization is also via the lung (Waldman and Ganguly, 1974), the immunological model used here is only marginally applicable to the infectivity model.

In order to directly relate the immune system to the infectivity model, it will be necessary to design experiments to test for pollutant effects on the pulmonary immune system, both as it exists as a steady state level and as it can be enhanced by immunization. The immunosuppression reported here should be interpreted in terms of a pollutant effect on a systemic host defense mechanism, a potentially hazardous event

considering the total function of the immune system. It seems possible, however, that metals capable of affecting the systemic immune system could also alter pulmonary immune functioning.

The in vitro study that was conducted on a single component of pulmonary immune mechanisms, i.e. the Fc receptor of AM, did follow the trends seen in the infectivity model. Cadmium chloride inhibited the Fc receptor more than NiCl_2 . This would be expected to result in a decreased binding of opsonized bacteria to the AM and hence, decreased phagocytosis and increased opportunity for bacterial growth in the lung.

Given the predilection of various forms of Cd, Ni, Mn, V, Cr, and Pb for depressing host defense mechanisms, it is important to continue toxicity studies of these compounds. It is also advisable to continue the development of more sensitive techniques to further investigate the toxic effects of these metals, as well as other materials of environmental concern. Since the model systems described in this paper have proven effective for studies of both oxidants and metals, it seems advisable to use them for other ubiquitous pollutants.

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CANINE HEPATOTOXIC RESPONSE TO THE INHALATION
OF 1,1-DIMETHYLHYDRAZINE (UDMH) AND
1,1-DIMETHYLHYDRAZINE WITH DIMETHYLNITROSAMINE (DMNA)

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During a 6-month inhalation experiment conducted in 1974-75 (MacEwen and Vernot, 1975), it was found that dogs exposed to 5 ppm UDMH on a 6 hour/day, 5 day/week schedule developed significantly elevated serum glutamic pyruvic transaminase (SGPT) levels by the fourth week of exposure. Throughout the duration of the exposure, SGPT values for exposed dogs were stable at levels 3-4 times those of the control group. A sharp reduction, approximately 50%, occurred at 2 weeks postexposure. Recovery, however, was not complete by 11 weeks postexposure where the mean SGPT value remained significantly elevated above control and nearly the same as the 2-week postexposure results. Liver function tests were performed on dogs at exposure termination and at 4, 8 and 11 weeks postexposure. Prothrombin time and cephalin flocculation values were normal but bromsulphalein (BSP) measured in blood samples from exposed dogs showed significant retention at all time periods except the last, 11 weeks postexposure. BSP values for all exposed dogs were at normal levels. Histologic information relative to the extent of liver damage or change caused by UDMH exposure was desirable but not obtained in that the main purpose of this experiment is to determine the oncogenic capacity of UDMH exposed animals during their lifetime. All dogs are being maintained at Brooks Air Force Base, and are in good health. Liver enzyme values were normal by 27 weeks postexposure.

The strong evidence of hepatotoxicity revealed by SGPT and BSP measurements in the oncogenic study was surprising since UDMH had not been shown to be hepatotoxic by other investigators conducting acute and subchronic studies. Because the UDMH used for

the oncogenic study described previously contained approximately 0.12% dimethylnitrosamine (DMNA), a known liver toxin (Magee and Barnes, 1956 and 1967; Barnes and Magee, 1954; Terracini et al., 1967), the possibility existed that the DMNA was responsible for hepatotoxicity as reflected by increased SGPT levels in dogs exposed to 5 ppm UDMH. Accordingly, to answer this question rapidly a series of short-term inhalation experiments were conducted using beagle dogs.

In the experiments reported here, 2 male and 2 female beagles served as test subjects and were exposed in a Rochester Chamber. Two male and 2 female beagles served as controls and were exposed to air only in an adjacent Rochester Chamber. Both chambers were operated with nominal airflows of 30 cfm at a slightly reduced pressure to prevent leakage of the test chemical into the laboratory atmosphere. Analysis of chamber atmosphere was continuous, utilizing an AutoAnalyzer colorimetric technique. This is the same method used for monitoring UDMH in the 6-month inhalation study mentioned previously (MacEwen and Vernot, 1975). Blood samples were drawn from all dogs at biweekly intervals, or more often, and clinical determinations made for the following battery of tests:

RBC	Calcium
WBC	Glucose
HCT	Total Protein
HGB	Albumin
Differential Cell Count	Globulin
Sodium	SGPT
Potassium	Alkaline Phosphatase.

Bromsulphalein (BSP) retention times were determined at the beginning and end of the study. Baseline measurements were available for all dogs for at least 2 months prior to this study, thus insuring selection of healthy animals with stable blood parameters.

During the course of the experiments, liver wedge biopsies were taken from all dogs for pathologic examination. At the conclusion, all dogs were sacrificed and major organs submitted for gross and histopathology.

In the first and second experiments of the series, the dogs were exposed to 5 ppm UDMH determined to be free of DMNA by mass spectrometric analysis. In the final test, the dogs were exposed to a mixture of 0.12% DMNA in UDMH which gave an air concentration of an estimated 6 ppb DMNA and essentially 5 ppm measured UDMH.

The first exposure was conducted for 8½ weeks on a 6 hour/day schedule, omitting weekends and holidays. This was a duplication of the exposure regimen used in the 6-month study. As seen in Table 1, there were no elevations in mean SGPT values for the exposed dogs. All other clinical chemistry determinations were normal when compared to control values. To examine the possibility that 5 ppm UDMH (free of DMNA) may have caused liver changes not revealed by SGPT measurements, liver wedge biopsies were taken from all exposed and control dogs for pathologic examination at the conclusion of 8½ weeks of exposure. Histopathologic differences between exposed and control tissues were marginal, but it appeared that cytoplasmic degenerative changes in liver cord cells were greater and occurred more frequently in exposed animals. There was also a modest increase in the amount of yellow-brown granular material seen accumulated in Kupffer's cells of exposed dog tissue.

TABLE 1. MEAN SGPT VALUES OF DOGS EXPOSED TO
5 ppm UDMH¹ AND CONTROLS

<u>SAMPLE PERIOD</u>	<u>MEAN SGPT VALUE (INTERNATIONAL UNITS)</u>	
<u>Preexposure</u>	<u>Exposed²</u>	<u>Control²</u>
2 Months	36.8	30.0
1 Month	25.8	24.0
1 Week	31.5	30.0
<u>Intermittent Exposure</u>		
2 Weeks	37.8	32.8
4 Weeks	37.8	34.0
6 Weeks	32.0	29.0
8½ Weeks	36.8	32.0
<u>Before Continuous Exposure</u>		
	48.9	45.1
7 Days Continuous Exposure	41.5	37.5
13 Days Continuous Exposure	44.8	30.1

¹Free of DMNA by mass spectrometric analysis.

²N = 4.

The dogs were rested 5 days following surgery after which continuous exposure to 5 ppm UDMH (free of DMNA) was begun to see whether this would cause SGPT changes in exposed animals. Results of SGPT determinations made after 7 days of exposure and at the conclusion, 13 days, are shown in Table 1. The data in this table clearly show that intermittent exposure for 8½ weeks followed by continuous exposure for 13 days to 5 ppm UDMH (free of DMNA) does not elevate SGPT levels in dogs.

In the final test the female control dogs were placed in the exposure chamber and the female exposed dogs were placed in the control chamber. This interchange was made to take into account the possibility of sensitization of the dogs by exposure to UDMH before this final experiment began. This experiment was started within a few hours after the cessation of the previous one and the dogs were exposed for 16 days continuously to the mixture of UDMH and DMNA. As seen in Table 2, there were significant SGPT elevations in blood samples taken from exposed dogs after 10 and 16 days. Noticeable is the 25% increase in mean SGPT values from 10 through 16 days of exposure. BSP determinations made on all dogs at exposure termination showed no significant differences between exposed and control mean values, nor any trend toward elevations in individual values for exposed dogs. This study was concluded and all dogs were sacrificed and submitted for gross and histopathologic examination. Gross results revealed nothing more than lesions obviously from the liver biopsies taken after 8½ weeks of intermittent exposure to UDMH only. Histopathology results were, surprisingly, the same for exposed and control dogs. Hepatocytes were relatively uniformly pale and swollen although this alteration was slightly more prominent in the periacinar regions. The cytoplasm contained many eosinophilic granules as well as some yellow-brown pigment granules. The latter were also noted in Kupffer cells. Several nonspecific eosinophilic intranuclear inclusions were also seen.

TABLE 2. MEAN SGPT VALUES OF DOGS CONTINUOUSLY EXPOSED TO
5 ppm UDMH WITH 0.12% DMNA¹ AND CONTROLS

<u>SAMPLE PERIOD</u>	<u>MEAN SGPT VALUES (INTERNATIONAL UNITS)</u>	
<u>Days of Exposure</u>	<u>Exposed²</u>	<u>Control²</u>
10 Days	68.8**	42.3
16 Days	85.8**	37.5

¹Determined by mass spectrometric analysis.

²N = 4.

**Higher than control mean value at the 0.05 level of significance.

It appears from the results of these experiments that DMNA was the active agent producing increased SGPT levels but at the level tested insufficient to cause discernible hepatocellular changes or alterations in liver function as measured by BSP determinations.

In that DMNA is a known potent hepatotoxin and proven carcinogen in animals, the results of the 6-month UDMH (containing DMNA) chronic inhalation exposure and the subsequent assessment of oncogenicity during the animals' lifespan should be considered carefully to avoid unwarranted incrimination of UDMH.

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AMRL-TR-76-125

PAPER NO. 10

INTERACTION OF CARCINOGENS AND LUNG IRRITANTS

Sidney Laskin

New York University Medical Center
New York, New York

Due to Mr. Laskin's untimely death, the text of this presentation is not available for publication.

OPEN FORUM

DR. WILLOUGHBY (Johns Hopkins Hospital): I would like to ask Ms. Graham if she has looked at the effects of cadmium and nickel on the local immune response of the lung. That is, instilling erythrocytes in the lung and then lavaging it, looking at the foamy cells because this might be even more striking.

MS. GRAHAM (Environmental Protection Agency): No, we haven't as yet, but we are making plans to do that. We are trying to design an experiment that will examine the pulmonary immune system.

DR. GRANETT (University of California, Riverside): Dr. Leong, I wanted to comment that we are doing some work on a dust generator, and we will be reporting on it Friday, similar to the one that you said was developed in Minnesota. From the diagram you showed, we think ours probably does a better job. I have one question about your large chambers. Do you find that you have any hot spots in them or do you have good mixing within the chamber?

DR. LEONG (International Research and Development Corporation): As far as the Dow chamber is concerned, I have no direct data on hand. But I understand they have measured concentrations of test gases in different positions within the chamber and found it to be satisfactory. As far as the other chamber arrangement is concerned, I have had the experience of using a similar one with the exhaust lines located along the two sides of the chamber. My experiment is with a smaller chamber, 5 feet³ instead of 8 feet³. We generated a concentration of chloro methyl methyl ether and measured the CMME in the four corners, the center, the bottom, and several other positions up and down. We found it quite nicely distributed. The concentration for CMME was 10 ppm.

DR. BUS (University of Cincinnati Medical Center): I have a question for Judy Graham. In view of the reported Yugoslav data on the health effects of manganese aerosols and the fact that apparently there will be widespread use of manganese gasoline additive as a substitute for lead alkyls, I wondered if you have any plans for studies to evaluate manganese health effects?

MS. GRAHAM: Yes, our plans are fairly extensive. In fact, we just responded to a freedom of information request on that subject. What I presented on our manganese work was very recent and that's why there are so few data points. We're just beginning this study. We've got daily exposures of animals going for various oxide forms of manganese. We're beginning with the infectivity models and then we'll proceed to do studies such as we've discussed today. We're going to be looking at the immune system, growth of bacteria within the lungs, and depending on what we see, we'll be looking at other parameters, too.

MR. VERNOT (University of California, Irvine): I'd like to ask Dr. Witschi how he came to choose BHT as the lung irritant before treating the animals with high levels, high partial pressures of oxygen. I've always thought that BHT might have a protective effect against oxidation being used as an antioxidant.

DR. WITSCHI (University of Montreal): When I became interested in lung biochemistry, I didn't have inhalation facilities so I was looking for compounds which cause pulmonary changes and which I could get simply by intraperitoneal injection. BHT turned out to be an agent which induces cell proliferation in lung. To answer the second part of your question, why it does not protect against oxygen is simply because about two days after injection of BHT, there is not much left in the lung so it can't act as an antioxidant. What I did was use BHT as a tool to produce cell proliferation in the lung and then look for the effects of oxygen on cell proliferation, like you would for example, study the effect of a drug on cell proliferation on a partially hepatectomized animal. BHT was just a tool to get the effect.

DR. SMITH (National Institute for Occupational Safety and Health): Dr. Laskin, do you have any information, using your protocol, on the inhalation effects of submicron sizes of fibrous glass?

DR. LASKIN (New York University Medical Center): No, we haven't anything that I can add other than what's been published. There is very little information on this subject; the implant approach of the people at NCI, and Smith at Fairley-Dickinson has been doing similar studies but, otherwise, there is very little data on the small fibers.

DR. CROCKER (University of California, Irvine): Dr. Laskin, I noticed that you have been continuing to use some of the installation techniques in addition to inhalation exposure. Yet, you are one of those who originally brought forward the aerosol approach as a more close approximation of natural exposure. As the need for more inhalation carcinogenesis data continues to grow, do you anticipate using more and more installation applications for carcinogen testing than you do inhalation? My reason for asking is that I believe that Dr. Phalen, for example, made a very good point on the significance of particle size and many factors relating to deposition and clearance by which the natural exposure has a complexity that is not mimicked by installation, yet installation is cheap and quick.

DR. LASKIN: Well, you've answered the primary reasons for using it.

DR. CROCKER: What are you doing? Do you foresee that installation is going to be a screening approach?

DR. LASKIN: Well, I would prefer to use inhalation alone. I've preached this for many years back. We have used installation because of time expedience. We wanted to get some reinforcement data on the SO₂/BP study and the hamster approach was easy and available. I have two or three technicians who are expert at this technique. It was easy to use this technique for that and some of our other problems. We use a special isolation chamber for small experiments. We don't have many of these. We only have two for rodents, and we have some large ones which happen to have been tied up with some other experiments for the past two years. These experiments are rather expensive when you start talking of isolation. For those that don't know, essentially it's a system whereby you handle animals at a small chamber located within a dry box and treat everything much like you handle radioactive material with as many remote controls as you can afford, neoprene gloves to reach in and move an animal if you have to. Ideally, one would like all sorts of automation. But this gets very expensive. We only have two such systems and they are usually tied up. If we had more, we would do only inhalation. I totally agree with Dr. Phalen's approach. That's important.

DR. CROCKER: I guess I answered my question partly because I foresee a trend. And I'm wondering if there is any way we can offer suggestions as to whether that trend should go towards installation techniques.

DR. LASKIN: There are several kinds of questions we are asking. First, is a chemical carcinogenic by this route? Intratracheal injections are artificial. Dr. Cushman gets very unhappy with many of the experiments using intratracheal installation because one gets more peripheral tumors and there are also more multiple foci, which is not like the normal picture. He's always worried about paralleling what he sees in man. He's a working pathologist and has always been. For many years, when he ran Bellevue Hospital Pathology Department, he saw every lung cancer and would sit in conference with several and compare animal data versus human tumors. They had to agree. That does not mean they always agreed because nobody as far as I know has a good model for oat cell carcinoma. We recognize all the defects. But if we are going to ask the question, does something happen as the first step or doesn't it, at least it tells us something. It's going to happen. And then we may proceed to the inhalation experiment. I don't think we can satisfy government regulatory agencies or industry until we do the inhalation study. And this is rather important. And I will point this out in a different way. When Ben Van Duren did animal skin painting studies on bis chloromethylether and said he got positive results, reported this, published it, nobody paid any attention. When we reported the results of the inhalation studies, everybody listened, including the Surgeon General of the United States, and the wheels began to turn. Because this response was like what people got. In many cases, as Dr. Weisburger said, some of these things are nice blinking yellow lights. Let's go on to such as mutagenesis. In our case where we studied bis chloromethylether, we got skin mutagenesis and finally carcinogenic changes by inhalation. In a new material, MCC, skin mutagenesis and inhalation induced changes fitted which indicated, certainly in these cases, that the preliminary mutagenic tests were good indicators of the carcinogenicity of this compound. I know of one compound we're working on now where it may not be true. The preliminary tests are positive and the inhalation studies may be negative. It may be that metabolic clearance is too good for this compound.

DR. HENDERSON (Olin Corporation): This may be nit-picking to a certain extent. We are trying to duplicate an industrial exposure of 6 hours a day, 5 days a week with a species of animal that is a nocturnal animal. Therefore, we are exposing our animals during the time that they normally would not have interaction with their dietary intake. Is anybody thinking of running their exposures on these rodent species at nighttime? That question is for anybody on the panel.

DR. LASKIN: I might comment that a number of us thought about shifting the day/night cycle of exposure. Unfortunately, you know, we all have to go back to somebody who pays the bills and we've gotten negative responses. I've been arguing for some years that circadian rhythms are important and that's part of the problem. But I haven't been able to sell this concept. I'd be glad to have industry help sell it. I want to point out something rather important about this sort of thing here. The rodent tends to go to sleep during the day and he's at a rest phase. Turn the lights out, turn the red light on, at least in mice I know for sure, they can't see it so it's night. Suddenly it's a different world full of activity. Some years ago I was involved with Herb Stokinger in an experiment conducting hydrogen fluoride exposures. Essentially nothing had happened at one particular level that we had used, and then we put the animals in activity cages to force them to be active. It was a totally different world. If I remember correctly, the rats were all killed at a level that would have been taken essentially as innocuous. We've got a long way to go in this direction, no question about it.

DR. S. MURPHY (Harvard School of Public Health): I might comment on some experience that we've recently had in our laboratory with vinylidene chloride. There appears to be a very striking difference in its acute hepatotoxic action between exposure in the morning and in the evening. This may be a circadian cycle but we don't know whether it's related specifically to the time of day or the feeding cycles of rats. We are not quite positive yet, but there appear to be some really very striking differences of several fold in the effective inhalation dose depending on whether they are exposed during the day or in the evening. As I recall, the evening exposure caused a greater response.

DR. LASKIN: Were the laboratory lights on during this experiment?

DR. S. MURPHY: Do you mean had the animals been adapted to a change in the lighting schedule? The answer is no. During the evening exposure, the lights were on. The animals were in a lighted room in other words. But they were not eating at the time anyway.

DR. RASMUSSEN (University of California, Irvine): I have a question for Dr. Laskin or anyone else who would care to comment. Do you have any feeling as to which class of cells within the lung is giving rise to the tumors in your experimental systems? I think there was some evidence for the type 2 cells giving rise to some human tumors but how about in the experimental animals?

DR. LASKIN: Well again, what is the origin of the tumor? If you are talking bronchogenic origin, generally one suspects the precursor or septal cell, the primitive cell because these are very rapidly growing. You can injure the cell and in as little as four days, you can see regeneration. This is *in vivo*. I'm not even talking about culture. In culture, in ciliated mucosa growth, the cilia grows back even more rapidly. You slice a bronchial or tracheal ring and place it in culture media and in a couple of days, it's just surrounded with ciliated cells. So there's a very rapid turnover from these primitive or embryonic cells which are part of what makes up the pseudostratified look of the epithelium. Now, if you talk about something originating in the alveolar sacs, then this is controversial. Perhaps Dr. Crocker could answer whether this is originating from type 2 cells. There is another question of whether it's bronchiolar or alveolar. In other words, are the cells really moving down and taking over as it appears in some cases.

DR. WITSCHI (University of Montreal): The lung adenoma in the mouse has been unequivocally shown to derive histogenetically from type 2 alveolar cells but that's only in the mouse. There has recently been a great interest in the biochemistry of the type 2 cells. People are devising all kinds of methods to study isolated type 2 cells. One way to do this is to harvest the type 2 cells from a urethane induced adenoma in the mouse. Also in about 1970, a cell line was established at NCI that was derived from a human alveolargenic carcinoma. Recently, these cells have been analyzed biochemically and their biochemical pathways, their surfactant synthesis, and all the enzymes involved in this resemble very much what we know is going on in normal type 2 alveolar cells. There is at least this particular type of a human tumor derived from type 2 alveolar cells. But as far as I understand, this human tumor is rather a rare one.

DR. CROCKER: There has been a lot of discussion about the tumors that appear in the alveoli of rodents as squamous carcinomas after either inhalation or intubation exposure. Many of these tumors do have an associated previous epithelialization of the alveoli by columnar cells which do not differentiate to ciliated forms very often, but even that may sometimes appear. This raises the question of whether or not the original damage to the alveoli wiped out the native epithelium permitting the epithelium from higher in the respiratory tract to grow down into and invest the inferior of the alveoli with the subsequent potential for conversion to a squamous state similar to that potential for conversion to a squamous state that exists in the trachea or major bronchi. The possibility, therefore, that there is a population of cells in the upper respiratory tract that can be converted to a squamous carcinoma, and that this cell population can be accounting for the squamous carcinomas that occur in the alveolar zone of the rodent lung following these heavy exposures seems to be fairly well supported. But that stands in some contrast with the alveolar adenoma of the mouse which occurs by administration of systemic carcinogens and which also does undergo in time a squamous carcinomatous alteration. Thus, we still are left with the issue unresolved, whether the type 2 cell is a brother of the epithelial cell of the larger airways, both of which are capable of conversion to a squamous state, or whether the squamous carcinoma depends upon some invasion from the upper respiratory epithelium.

DR. WITSCHI: I would like to ask whether the adenoma in the mouse lung is considered to be a benign tumor. Is that correct?

DR. CROCKER: I believe you are correct. The adenoma of the mouse is in general a benign tumor. But in very old animals, some of these tumors may undergo spontaneous carcinomatous changes. Often when that happens, these are squamous carcinomas. Now, I'll stand corrected if anyone cares to challenge that. They are clearly type 2 adenomas in the strain A mouse lung.

DR. WITSCHI: I would like to have more information about where the cells or these tumors come from. When I learned that BHT could produce type 2 cells that proliferated and would go into division, it occurred to me that it would be possible, with repeated injections, to promote urethane carcinogenesis because you hit the same target cells. BHT in itself is not carcinogenic, but with one single dose of urethane, followed by weekly injections of BHT, produces more tumors than urethane alone.

DR. MURPHY: We haven't heard much on a few issues that I thought were just touched upon in the discussion this afternoon. Dr. Leong mentioned good laboratory practices as part of his discussion and I'm sure we are all aware of the controversy developing with respect to "good laboratory practice." I wonder if regulatory actions or formal regulations are going to require greater standardization of the equipment and methodology in the inhalation toxicology research laboratory. Would you have any feeling on that, Dr. Leong?

DR. LEONG: I'm not sure about the inhalation toxicology laboratory but I'm prepared for it. As for the other laboratory techniques such as feeding experiments and the other routes of administration, I am sure that the necessity of improvement is coming. I have a very, very strong objection to being told what equipment and what procedures to use. One of the great strengths of this field of toxicology is that none of us is happy with what goes on. We always want to build a little bit better system. And that's great. Because that means we're in there thinking and fighting. And we do a little bit better science. I'm not talking about just equipment. I'm talking about the overall methodology. If we are boxed in with regulated methods then we are working out of a cookbook. I'll go look for another job and so will many of you because you wouldn't be happy.

AMRL-TR-76-125

SESSION III

GENERAL TOXICOLOGY

Chairman

Col. Marshall Steinberg, MSC
Laboratory Sciences Consultant
HQDA (DASG-HCC-L)
Room #2D528, The Pentagon
Washington, D. C.

THE EFFECTS OF ETHYL PARATHION IN THE RAT AND DOG AFTER
ACUTE AND SUBACUTE INHALATION AND ORAL ADMINISTRATION

Edmund J. Owens

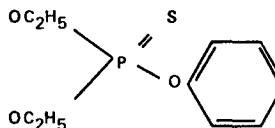
Department of the Army
Edgewood Arsenal
Aberdeen Proving Ground, Maryland

INTRODUCTION

Parathion is an "organophosphorus" insecticide developed by G. Schrader in 1944. It has a molecular weight of 291.27. The chemistry and mechanism of biological action are illustrated in Figure 1.

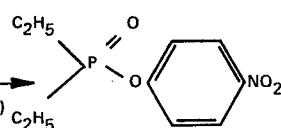
CHEMISTRY

PARATHION (WEAK ChE INHIBITOR)



INDIRECT ChE INHIBITOR

PARAOXON (STRONG ChE INHIBITOR)



DIRECT ChE INHIBITOR

MECHANISM OF ACTION

PARAOXON - POTENT INHIBITOR OF RBC & PLASMA CHOLINESTERASES

RESULANT PHOSPHORYLATED ENZYME STABLE - SLOW REACTIVATION BY HYDROLYSIS

HYDROLYSIS (REACTIVATION) ALSO LIMITED BY AGING

AGING - SPONTANEOUS REACTION LEADING TO STABLE PHOSPHORYLATED RBC ChE ATTRIBUTED TO MONO DEALKYLATION OF THE PHOSPHORYL OR PHOSPHONYL MOIETY, CHANGING ELECTRONIC CHARGE OF THE PHOSPHOROUS ATOM SUCH THAT IT CANNOT BE APPROACHED BY THE HYDROXYL ION OF WATER

Figure 1. Biological effects of exposure to parathion.

Parathion is highly toxic to mammals and should not be used by man unless necessary safety precautions are observed and personal protective equipment is used. The parathion hazard is

directed toward personnel employed in agriculture, especially those who handle the insecticide and are involved in spraying. Employees of industrial plants where parathion is packaged and synthesized should also be aware of the hazard.

The National Institute of Occupational Safety and Health required additional acute and subacute toxicity data on rats and dogs exposed by the oral and inhalation routes in order to establish safety criteria for ethyl parathion.

The effect of the insecticide on both erythrocyte (RBC) and plasma cholinesterase (ChE) activity in dogs and rats was requested. It is anticipated that these experimental results with animals would contribute to the prediction of safe levels for human exposure.

INHALATION TOXICITY OF ETHYL PARATHION

MATERIALS

A technical grade of ethyl parathion, made by the Monsanto Company, was used in these tests. The diazo assay of the compound was 99.33%.

Adult male colony rats (Sprague-Dawley crossed with Wistar) and purebred adult male beagle dogs were used in the inhalation tests.

METHODS FOR CHOLINESTERASE DETERMINATION

The blood samples from rats and dogs exposed to ethyl parathion were analyzed by Technicon AutoAnalyzer method N17P. Each sample as received was spun down in an International Portable Refrigerated Centrifuge at 2000 rpm (approximately 700 G) for 20 minutes at 4 C. The red cells and plasma were separated, and 0.4 ml of each was diluted to 2.0 ml with saline. When 0.4 ml of red cells or plasma was not available, a lesser volume was used, but the proportions of sample to saline diluent were kept at 1:4.

The activity was measured in Deca International units of thiocoline formed by enzyme per liter at 37 C under the operating conditions for method N17P. The International Union of Biochemistry (IUB) defines 1 unit of enzyme activity as the amount of enzyme that will catalyze the transformation of 1 micromole of substrate per minute under specified reaction conditons. Ten IUB International Units equal 1 deca unit.

The stock solution is equivalent to a sample of 90.9 deca units per liter activity; however, the sample was diluted 1:4 with saline and is only 20% (v/v) in red cells or plasma. The samples' original activity was 5 X (90.9) or 454.5. The analytical procedure is not accurate to four significant figures and the answer should be rounded off to 454 deca units per liter of undiluted red cells or plasma.

For quality control, paper strip controls were made up and run (method of Fleisher).

Normal serum of equine origin was diluted and run as a control periodically. This equine serum was obtained from the Pitman-Moore Company, Indianapolis, Indiana.

ACUTE INHALATION EXPOSURE OF RATS

Groups of 34 male rats were exposed to 20 aerosol concentrations of ethyl parathion (0.035 to 230.0 mg/m³) in a 1000-liter dynamic flow chamber for 4 hours.

During exposure the rats were observed for toxic signs and death. Blood samples for RBC and plasma ChE determinations were obtained from groups of six rats at 4 hours and 1, 2, 7 and 14 days postexposure. The following values were calculated: LC₅₀'s, EC₅₀'s, Et₅₀'s for toxic signs and ChE₅₀'s.

Baseline RBC and ChE levels were obtained from 71 unexposed male rats, and additional unexposed rats were used as controls for various concentration levels of parathion.

Chamber air samples were collected on two fiberglass filter pads. The parathion on the pads was diluted with isopropyl alcohol and analyzed by a gas chromatographic technique. Particle size (1.0-2.0 microns) was determined by use of a Rochester cascade impactor. The instrumentation and procedures for measuring ethyl parathion by gas chromatography are as follows:

Instrument. Micro-Tek Model MT 220 supplied by Micro-Tek Instruments Corporation, a subsidiary of Tracor, Inc., Austin, Texas 78721.

Detector. Flame Photometric Detector, Model FPD 100 with phosphorus filter supplied by Melpar, Inc., a subsidiary of Westinghouse Air Brake Co., Falls Church, Virginia.

Column. Six-ft, glass (OD 0.4 mm, ID 0.2 mm) 10% OV-1, Chromosorb W, 80/100 mesh supplied by Applied Science Laboratories, Inc., PO Box 440, State College, Pa.

Carrier Gas. Nitrogen, prepurified grade, supplied by Linde Gas Products.

Recorder. (1) Autolab System IV, B, Computing Integrator for chromatography supplied by Autolab, a division of Spectra-Physics, 655 Clyde Avenue, Mountain View, California 94401, and (2) Spectrum 1021 Filter and Amplifier supplied by Spectrum Scientific Corp., 2401 Ogletown Road, Newark, Delaware.

Syringe. 10- μ l Hamilton (#701) supplied by the Hamilton Co., Reno, Nevada.

Sample Size. 4.0- μ l with 1.0- μ l isopropanol pusher, and ca. 1.0- μ l air space in between.

Temperature. Injection port - 225 C
Column - 225 C
Detector - 160 C (max for this FPD set-up)

Flow Rate.

	<u>Pressure</u> (psig)	<u>Rotameter</u> <u>Setting</u>	<u>Flow</u> cc/min STP
Carrier gas, nitrogen	40	10.0	105
Oxygen*	40	25.0	NA
Hydrogen**	40	180.0	NA
Air**	40	30.0	NA

*Supplied by Linde Gas Products, prepurified.

**Supplied by Aberdeen Proving Ground.

Retention Time. Parathion - 4.6 minutes.

Procedures. A parathion reference standard solution was obtained from Monsanto Company on 15 May 1975 and had a purity by diazo assay of 99.33%. Using this standard, the following solutions were prepared in isopropanol.

<u>Experimental Concentration Level</u>	<u>Standard Concentration Level</u>
1.0 mg/liter	4.06 mg/ml
0.1 mg/liter	0.518 mg/ml
0.01 mg/liter	0.0518 mg/ml

The concentration of parathion in samples (mg/liter) was calculated as follows:

$$\frac{(\text{PA}_s)}{(\text{PA}_{\text{std}})} \quad \frac{(\text{Conc}_{\text{std}})}{(\text{Vol}_{\text{solvent}})} \quad \frac{(\text{Vol}_{\text{solvent}})}{(\text{Vol}_{\text{air}})}$$

PA_s = Peak area counts of sample

PA_{std} = Peak area counts of standard solution

Conc_{std} = Concentration of standard solution in mg/ml

$\text{Vol}_{\text{solvent}}$ = Volume of isopropanol for extraction in ml

Vol_{air} = Volume of sampled air in liters.

ACUTE INHALATION EXPOSURE OF DOGS

Groups of four adult male dogs were exposed to five concentration levels of parathion aerosol for 4 hours. The parathion concentrations were 0.0153, 0.145, 3.41, 8.93, and 37.13 mg/m³. The animals were observed for toxic signs during and after exposure. Blood samples were obtained from the dogs prior to exposure for baseline RBC and plasma ChE values. Additional blood samples were obtained from the dogs just prior to exposure and at 4 hours, 1, 2, 7 and 14 days postexposure for ChE determination.

SUBACUTE INHALATION EXPOSURE OF RATS

Groups of 80 male rats were exposed for 7 hours/day, 5 days/week for 6 weeks to three concentrations (0.01, 0.10, or 0.74 mg/m³) of parathion aerosol. The concentrations were selected on the basis of data gained during the acute exposures. To obtain baseline RBC and plasma ChE determinations, 71 rats were sacrificed to obtain samples. Three additional groups of 80 rats each served as controls at each concentration of parathion. At various times during the 6-week exposure period and during the 6-week observation period, groups of 10 control rats along with groups of 10 exposed rats were sacrificed. They were first observed for toxic signs, weighed, and their blood drawn.

SUBACUTE INHALATION EXPOSURE OF DOGS

Groups of six adult male dogs were exposed to aerosols of parathion (0.001, 0.01, or 2.0 mg/m³) for 7 hours/day, 5 days/week for 6 weeks. All dogs were examined by a veterinarian prior to use and declared to be free of infectious disease. The animals were held for observation for 6 weeks after exposure.

Blood samples were taken from each dog before exposure to obtain average RBC and plasma ChE values. Each dog also served as its own control. RBC and plasma determinations were also made from blood samples drawn at various times during the exposure and postexposure periods. All dogs were observed for toxic signs.

The experimental protocols for the acute and subacute inhalation and oral phases of the study are shown in Table 1.

TABLE 1. PROTOCOLS FOR ETHYL PARATHION STUDIES

PHASE	SPECIES	INHALATION			ORAL		MEASUREMENTS
		EXPOSURE CONCENTRATION	DURATION OF MEASUREMENT	DOSE	DOSE FREQUENCY		
		MG/CU M	HRS/DAY	MG/KG	DOSE /DAY		
ACUTE	RAT	<u>20 LEVELS</u> <u>(0.35 - 230)</u>		4/1	LC ₅₀	<u>5 LEVELS</u> <u>(4.0 - 10.0)</u>	LD ₅₀ Et ₅₀
					ChE ₅₀	<u>7 LEVELS</u> <u>(0.175 - 7.0)</u>	
					Et ₅₀	<u>1 LEVEL</u> <u>(2.8)</u>	ChE RECOVERY RATE
	DOG	<u>5 LEVELS</u> <u>(0.015 - 37.1)</u>		4/1	" "	<u>5 LEVELS</u> <u>(2.5 - 20.0)</u>	LD ₅₀
						<u>4 LEVELS</u> <u>(0.50 - 10.0)</u>	Et ₅₀
						<u>1 LEVEL</u> <u>(2.5)</u>	ChE RECOVERY RATE
SUBACUTE	RAT	<u>3 LEVELS</u>	7/30	ChE ₅₀	<u>3 LEVELS</u>	1/30	ChE ₅₀
		0.01	5 DA/WK		0.05	5 DA/WK	
		0.10	6 WKS	Et ₅₀	0.10	6 WKS	Et ₅₀
	DOG	0.74			0.25		
		<u>3 LEVELS</u>	7/30	" "	<u>3 LEVELS</u>	1/30	" "
		0.001	5 DA/WK		0.05	5 DA/WK	
		0.01	6 WKS		0.10	6 WKS	
		0.20			0.50		

RESULTS

Effects of Acute Inhalation Exposure in Rats

The LC₅₀ for male rats exposed for 4 hours to parathion aerosols is 84.00 (78.03 to 90.44) mg/m³. Toxic signs (tremors, convulsions, salivation, respiratory difficulty) and death were seen in rats exposed to concentrations ranging from 50.0 to 230.5 mg/m³. The EC₅₀ for tremors is 73.67 (67.15 to 80.83) mg/m³ and the EC₅₀ for convulsions is 11.06 mg/m³ (96.0 to 127.4).

The Et₅₀ values for tremors, convulsions and death were determined in rats exposed to concentrations of 97.0, 100.6, 118.5, or 230.5 mg/m³. Other observations noted at a concentration level of 28.08 mg/m³ were as follows: at 43 min, occasional sneezing; at 50 min, possible nose irritation; at 4 hr, approximately 15/34 had diarrhea and the scrotal area was wet with urine; and at 3.5 hr postexposure, lethargy and in a few animals "wet dog shakes."

Thirteen dose levels ranging from 0.04 to 35.0 mg/m³ were used for determination of RBC and plasma ChE values. The dose that inhibited 50% of the RBC ChE activity (ChE₅₀) was determined to be 5.43 (4.20 to 7.03) mg/m³. The ChE₅₀ for plasma was determined to be 7.28 mg/m³ (5.24 to 10.12).

Effects of Acute Inhalation Exposure in Dogs

Dogs in groups of four each were exposed to aerosolized parathion for 4 hours, one group each at levels of 0.0153, 0.145, 3.42, 8.93 and 37.13 mg/m³. Cholinesterase was significantly inhibited by all five concentrations. However, due to the high levels of depression seen in each group and the fact that lower concentrations could not be tested due to the shortage of dogs, calculations of ChE₅₀ values were not possible. No deaths occurred at any level tested.

Effects of Acute Oral Doses in Rats

In tests to determine the LD₅₀ of parathion, five groups of 10 adult male rats were given 4.0, 5.0, 6.3, 7.9 or 10.0 mg/kg of parathion diluted in corn oil in single oral doses administered by stomach tube. There were control groups for each dose.

Tests to determine the dose that inhibits 50% of the ChE activity were conducted using 80 adult male rats. Seven groups of 10 each received doses ranging from 0.175 to 7.000 mg/kg in a constant volume of 5 ml/kg and blood samples were taken 24 hours later. An additional group of 10 rats served as controls.

Sixty male rats were used to determine the recovery rate of RBC and plasma ChE activity following a single oral dose of 2.80 mg/kg of ethyl parathion; 20 rats received corn oil only and served as controls. Blood was taken from six groups of 10 rats each at 4, 24, 48, 72, 168 and 336 hours, respectively, after exposure.

The 24-hour LD₅₀ of ethyl parathion in the rat was found to be 6.85 (6.18 to 7.60) mg/kg. The toxic signs observed and the Et₅₀'s, ED₅₀'s, and the Lt₅₀'s for each group were determined.

The dose that inhibited 50% of the RBC ChE (ChE₅₀) was determined to be 2.579 (2.117 to 3.141) mg/kg and the plasma ChE₅₀ was 2.546 (2.123 to 3.045) mg/kg. After 336 hours, group average recovery of the RBC ChE activity was only 67% of normal while the plasma ChE was still 11% inhibited. These data were statistically evaluated to fit the actual data points.

Effects of Acute Oral Doses in Dogs

Five groups of four dogs each received 2.5, 6.3, 10.0, 15.8 or 20.0 mg/kg of ethyl parathion in corn oil (capsule form) in single oral doses to determine the 24 hour LD₅₀. There were control groups of each dogs.

Four groups of four dogs each received 10.0, 2.5, 1.26 or 0.50 mg/kg of ethyl parathion in corn oil (capsule form). Blood samples were drawn 24 hours after dosing to determine the ChE₅₀.

Four dogs were used to determine the recovery rate of RBC and plasma ChE activity following a single oral dose of 2.5 mg/kg of ethyl parathion. Blood samples were taken at 24, 264, 360, 696 and 864 hours after exposure.

The 24 hour acute oral LD₅₀ of ethyl parathion in the dog was 8.27 (4.79 to 14.29) mg/kg. Because of the limited number of dogs used to determine the LD₅₀ no statistical analysis of times to response was done. The RBC ChE₅₀ was found to be 1.497 (1.060 to 2.115) mg/kg and the plasma ChE₅₀ was 1.670 (0.942 to 2.960) mg/kg.

At 24 hours after exposure, RBC ChE was inhibited 64% and plasma ChE activity 59%. Near normal levels of RBC ChE activity were reached at 864 hours and of plasma ChE activity at 696 hours.

Table 2 shows the acute inhalation and oral toxicities of ethyl parathion in the rat and dog, and Table 3 and 4 illustrate the Bliss analysis of the toxic response data (effective time) 50's for tremors, convulsions and death versus dose and for the rat and dog; a summary of the acute ChE (cholinesterase) 50's are shown in Table 5. The RBC activity of rats and dogs exposed acutely by inhalation are illustrated in Figures 2 and 3. The plasma responses for the same animals are shown in Figures 4 and 5. The RBC recovery rates for rats and dogs dosed orally are illustrated in Figures 6 and 7.

TABLE 2. ACUTE TOXICITIES OF ETHYL PARATHION IN THE RAT AND DOG

SPECIES	ROUTE OF ADMINISTRATION	LC50	LD50	ED50'S (MG/CU M - MG/KG)	
		MG/CU M (4 - HR EXP) (95% C. L.)	MG/KG (95% C. L.)	TREMORS	CONVULSIONS
RAT	INHALATION	84.00 (78.00 - 90.40)		73.67 (67.15 - 80.83)	110.6 (96.0 - 137.4)
	ORAL		6.85 (6.18 - 7.60)	4.48 (4.48 - 4.48)	6.69 (5.98 - 7.84)
DOG	INHALATION	DOSES OF 0.015 TO 37.1 (NO DEATHS)		N/R	N/R
	ORAL		8.27 (4.79 - 14.29)	9.05 (N.L.)	10.69 (N.L.)

*ALL RESPONDED EXCEPT LOWEST DOSE

TABLE 3. SUMMARY OF ACUTE ET₅₀ VALUES FOR THE RAT

ROUTE OF ADMINISTRATION	DOSE	ET ₅₀ (95% C.L.)		
		TREMORS	CONVULSIONS	DEATH
	MIN	MIN	MIN	
INHALATION	MG/CU M			
	71.0	210.1(201.0-219.5)		
	97.0	173.4(163.7-183.6)	184.3(170.0-200.0)	218.7(201.5-237.3)
	100.6	159.4(144.5-175.8)	179.2(163.2-196.8)	237.0(200.5-280.2)
	118.6	158.9(144.2-175.3)	167.8(153.2-183.9)	208.2(189.0-229.4)
ORAL	230.5	103.9(96.2-112.1)	114.1(104.9-124.2)	129.5(121.2-138.3)
	MG/KG			
	4.0	-	-	-
	5.0	43.8(39.9-48.1)	-	-
	6.3	69.1(48.8-97.7)	-	-
ORAL	7.9	46.1(34.1-62.3)	75.3(45.1-125.7)	108.3(5.15-227.4)
	10.0	28.1(23.5-33.5)	33.1(25.8-42.4)	45.6(36.5-56.8)

TABLE 4. SUMMARY OF ACUTE ET₅₀ VALUES FOR THE DOG

ROUTE OF ADMINISTRATION	DOSE	ET ₅₀ (95% C.L.)		
		TREMORS	CONVULSIONS	DEATH
	MIN	MIN	MIN	
INHALATION	MG/CU M			
	0.015			
	0.0145			
	3.42	NONE OBSERVED	NONE OBSERVED	NONE OBSERVED
	8.93			
ORAL	37.13			
	MG/KG	MIN (RANGE)	MIN (RANGE)	MIN (RANGE)
	2.5	-	-	-
	6.3	55-60	85-178	195-ON
	10.0	90-97	93-98	120-ON
ORAL	15.8	27-58	18-120	30-120,ON

TABLE 5. SUMMARY OF ACUTE ChE₅₀'S FOR THE RAT AND DOG

ROUTE OF ADMINISTRATION	SPECIES	RBC-ChE ₅₀		PLASMA - ChE ₅₀
		RAT	DOG	
INHALATION	RAT	5.43 MG/CU M (4.20 - 7.03)	SIGNIFICANT DEPRESSION (0.015-37.13 MG/CU M)	7.28 MG/CU M (5.24 - 10.12)
	DOG			SIGNIFICANT DEPRESSION (0.015-37.13 MG/CU M)
ORAL	RAT	2.58 MG/KG (2.12 - 3.14)		2.55 MG/KG (2.12 - 3.05)
	DOG	1.50 MG/KG (1.06 - 2.12)		1.67 MG/KG (0.94 - 2.96)

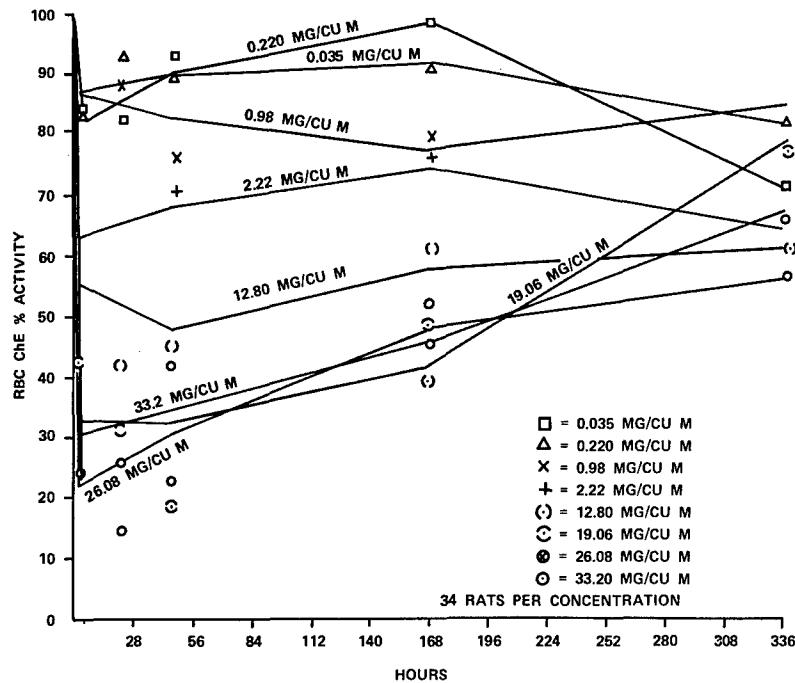


Figure 2. Red blood cell ChE activity in rats exposed to aerosols of ethyl parathion for four hours.

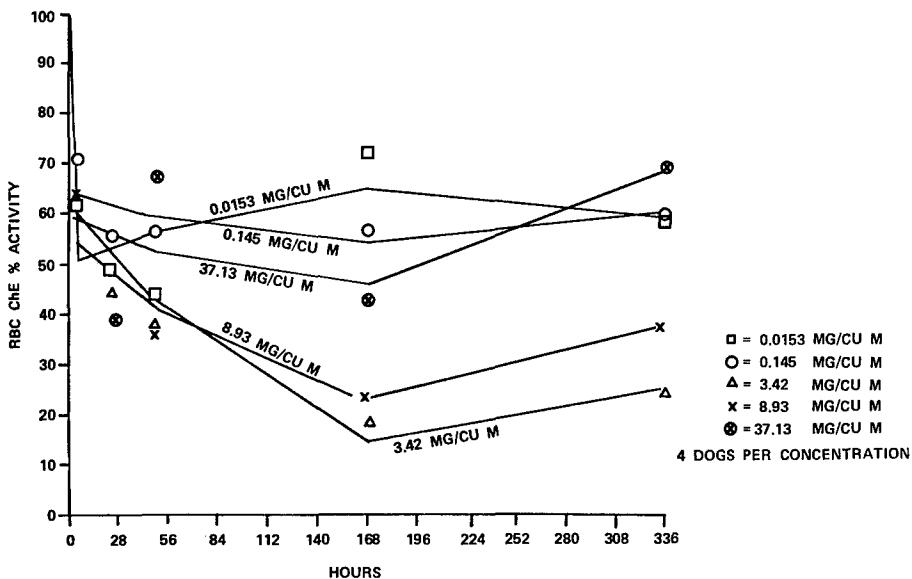


Figure 3. Red blood cell ChE activity in dogs exposed four hours to ethyl parathion.

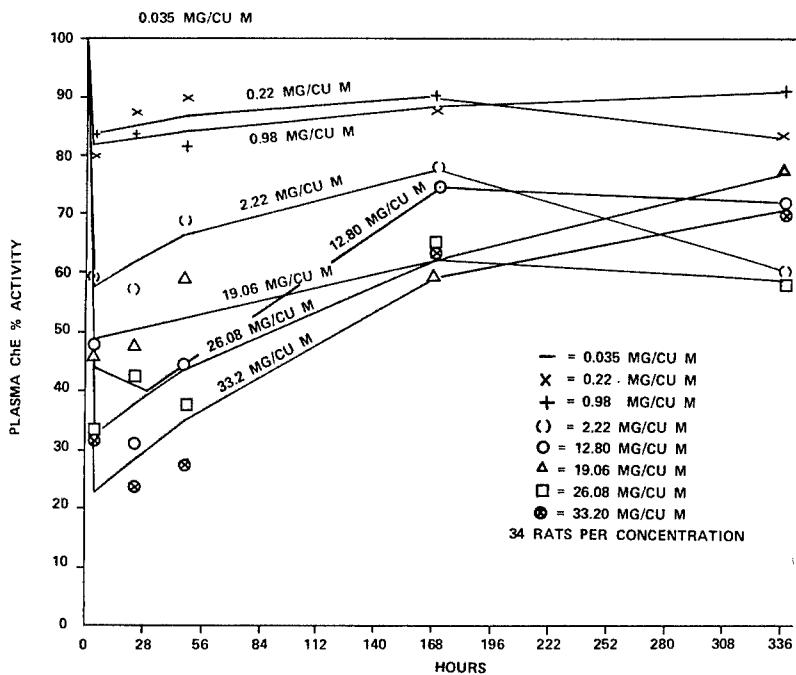


Figure 4. Plasma ChE activity in rats exposed to aerosols of ethyl parathion for four hours.

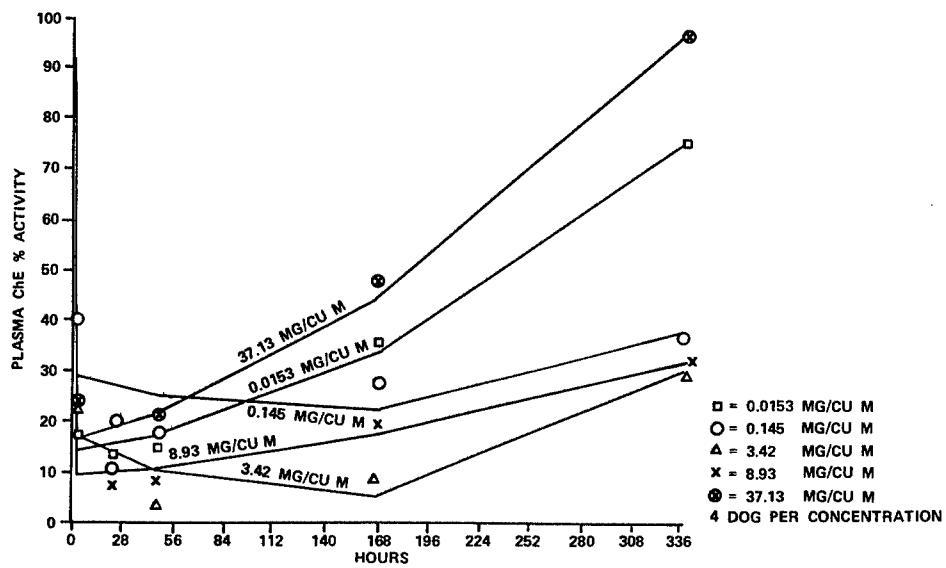


Figure 5. Plasma ChE activity in dogs exposed for four hours to ethyl parathion aerosols.

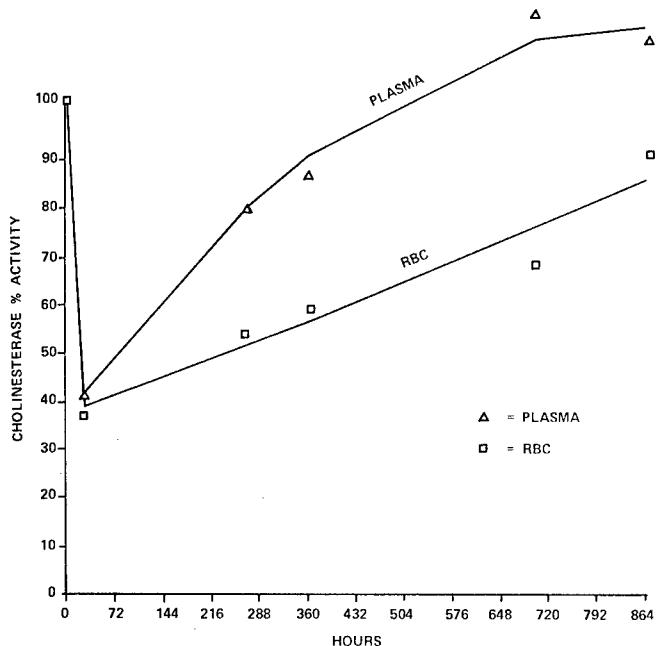


Figure 6. Recovery rate of ChE activity in rats exposed to a single oral dose (2.8 mg/kg) of ethyl parathion.

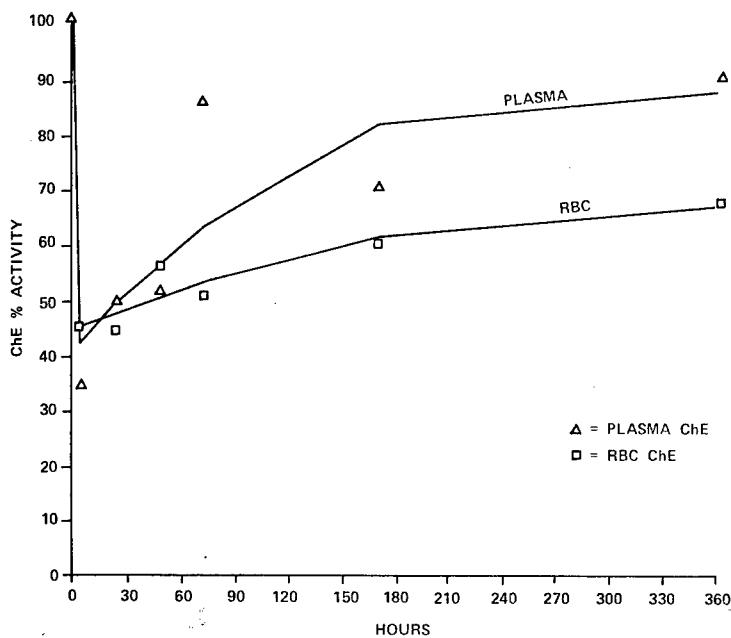


Figure 7. Recovery rate of ChE activity in dogs exposed to a single oral dose (2.5 mg/kg) of ethyl parathion.

Effects of Subacute Inhalation Exposure in Rats

The levels selected for testing were experimentally verified: the 0.01 mg/m³ concentration was a no-effect dose; the 0.10 mg/m³ level produced moderate effects; and 0.74 mg/m³ produced a pronounced effect.

No toxic signs were seen in the rats exposed to 0.01 and 0.10 mg/m³. However, one rat died on the first exposure day at the lowest level. Microscopic examination revealed lung congestion but no significant lesions due to the parathion exposure. One unexposed control rat from the 0.10 mg/m³ died on the 9th day; it had acute kidney, ureter, and bladder inflammation. There were no other significant lesions and the cause of death could not be definitely related to the action of parathion. At the 0.74 mg/m³ concentration, two rats died, one on the 10th day and one on the 28th day of exposure. Gross examination of the rat that died on the 10th day showed congested lungs, and edema was seen upon microscopic examination. This was an agent-related lesion. The rat that died on the 28th day had acute lung congestion, but no significant lesions.

Two rats exposed to the highest concentration were sacrificed due to their poor physical appearance. One rat, sacrificed on the 5th day of exposure, had severe malocclusion. The other rat showed tremors and ataxia 24 hours before sacrifice on the 15th day. This rat had escaped from its cage and was loose in the chamber.

Blood hematocrit values were obtained on four controls and nine test rats after the last exposure to the parathion concentration of 0.74 mg/m³. The values for the four controls were 47.8, 49.0, 51.0, and 45.9 vol %. The values for the nine exposed rats were 47.2, 44.9, 49.3, 47.8, 45.5, 47.3, 48.8, 47.8, and 46.6 vol %. The average was 47.2 vol % which is not significantly different from the control average.

The rats were weighed just prior to obtaining blood samples and during and after exposure. They gained weight throughout exposure to all three levels of parathion and also during the postexposure period.

Effects of Subacute Inhalation Exposure in Dogs

RBC and plasma ChE values (% activity) for dogs exposed to aerosol concentrations of 0.001, 0.01, or 0.20 mg/m³ of parathion were determined.

Red blood cell and plasma ChE activities tested at the 0.001 mg/m³ level remained essentially unchanged during the exposure and postexposure periods. This concentration was considered to be a no-effect dose. At the 0.01 mg/m³ concentration, ChE activity was 78.6% in RBC and 69.6% in plasma at the end of the 2nd week of exposure. This level was considered to have a moderate effect. The high dose, 0.1 mg/m³, could be considered as having a severe effect. By the end of the 2nd week, RBC ChE activity was 54.0 and plasma activity was 26.0; the RBC ChE activity did not return to normal until the 4th week of the postexposure recovery period. No other toxic signs were observed.

The Effects of Subacute Oral Doses in Rats

A total of 480 adult male rats were used to determine the effects of repeated daily oral doses of ethyl parathion. One half of the rats received daily doses of corn oil (5 days/week for 6 weeks), while the other 240 rats received daily doses of ethyl parathion in corn oil. Doses were given on a ml/kg basis. Control rats received 1 ml/kg of corn oil and the test animals received 1 ml/kg of corn oil containing ethyl parathion in concentrations of 0.25 mg/ml, 0.10 mg/ml, or 0.05 mg/ml. There were 80 rats per dose level in each test group and each control group. All rats were weighed daily.

Blood samples for ChE determination were taken from 10 control and 10 test animals at each dose level at 1, 2, 4 and 6 weeks during exposure and at 1, 2, 4 and 6 weeks postexposure if required.

No toxic signs were observed in any of the test or control rats during or after exposure. Weight gained by controls was not significantly different from that gained by the test rats.

The high dose (0.25 mg/kg) produced about a 55% inhibition in RBC ChE activity and 48% inhibition of the plasma ChE activity. No significant inhibition resulted from the 0.05 mg/kg dose

while the 0.1 mg/kg dose produced about 20% inhibition. The rats that received the 0.25 mg/kg dose recovered normal ChE levels at 6 weeks after exposure, while the rats that received the two lower doses recovered 1 week after exposure.

Effects of Subacute Oral Doses in Dogs

Twenty-four adult male dogs were given oral doses (0.05, 0.1, or 0.5 mg/kg) of ethyl parathion in solution with corn oil in capsule form 5 days/week for six weeks. In each group six dogs were given oral doses of parathion and two dogs were given corn oil only. Doses were given on a mg/kg basis. The dogs were weighed weekly. No toxic signs were observed in any of the dogs during or after their exposure to these doses of parathion.

After 6 weeks, the 0.50 mg/kg dose produced an approximate 58% inhibition of RBC ChE and 85% inhibition of the plasma ChE activity; recovery 6 weeks after exposure was incomplete with 33% inhibition of RBC ChE and 26% inhibition of the plasma ChE. Inhibition from the 0.10 mg/kg and 0.05 mg/kg doses of parathion were similar. The 0.1 mg/kg dose did inhibit ChE activity slightly more but the recovery rates for both doses were very close. Near normal levels of ChE activity were reached 2 weeks after exposure.

The RBC activities in rats and dogs exposed subacutely to aerosols of parathion for 6 weeks are illustrated in Figures 8 and 9. The residual RBC activities in rats and dogs dosed subacutely for 6 weeks are shown in Figures 10 and 11.

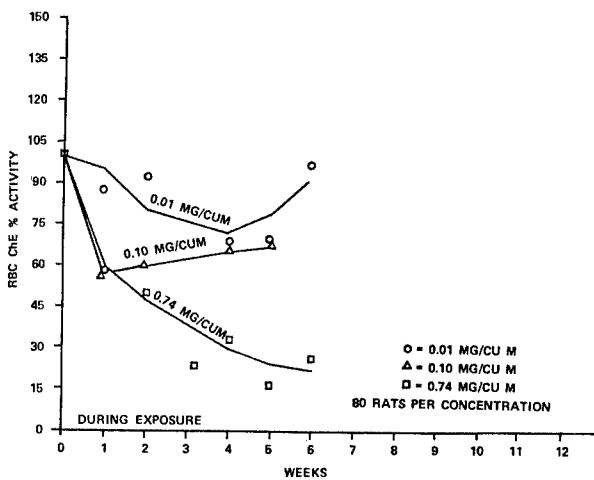


Figure 8. RBC ChE activity in rats exposed subacutely to ethyl parathion aerosols.

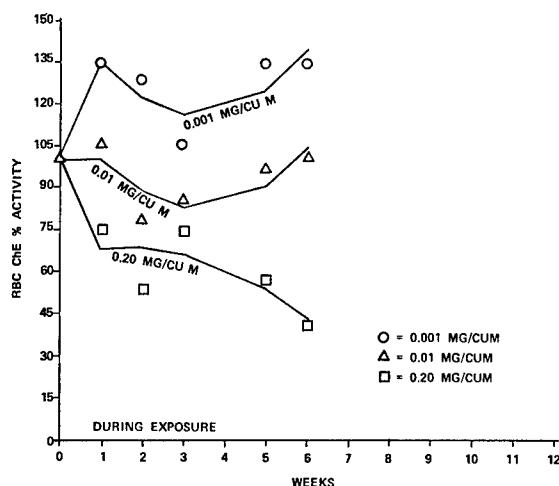


Figure 9. Red blood cell ChE activity in dogs exposed subacutely to aerosols of ethyl parathion.

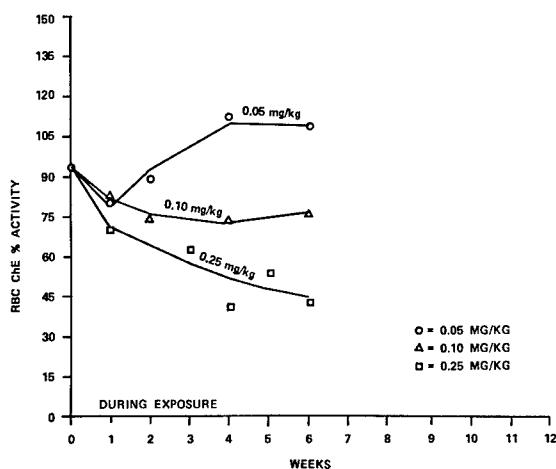


Figure 10. Residual RBC ChE activity in rats given subacute oral doses of ethyl parathion.

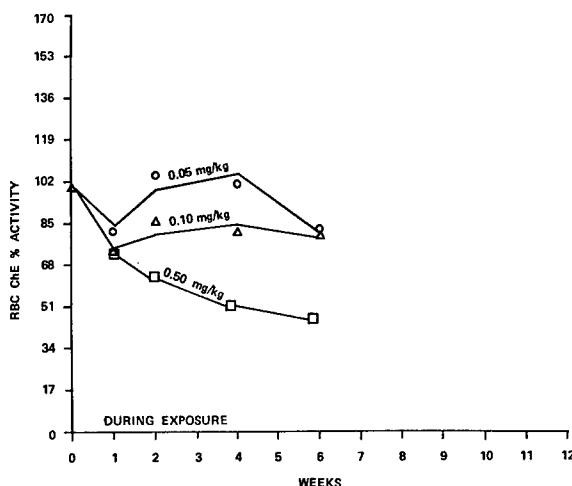


Figure 11. Residual RBC ChE activity in dogs given subacute oral doses of ethyl parathion.

SUMMARY OF RESULTS

Inhalation

The plasma ChE₅₀ for rats exposed for 4 hours to 13 levels of ethyl parathion aerosol (0.035 to 35.0 mg/m³) is 7.28 mg/m³ with 95% confidence limits of 5.24 to 10.12; RBC ChE₅₀ is 5.43 (4.2 to 7.03) mg/m³. For rats exposed to eight levels of ethyl parathion (31.36 to 230.5 mg/m³) the LC₅₀ is 84.0 (78.9 to 90.4) mg/m³.

In rats exposed subacutely to 0.01, 0.10, or 0.74 mg/m³, these concentrations, respectively, produced no effects, moderate effects and pronounced effects on ChE activity. The RBC ChE activity in rats exposed to the highest dose did not return to normal until the 6th week after exposure. In two deaths that occurred at the high dose, microscopic examination revealed an agent-related lesion of the lungs in one rat; the other had acute lung congestion but no significant lesions.

In the acute inhalation tests of dogs exposed for 4 hours to five dose levels ranging from 0.0153 to 37.13 mg/m³, there was a pronounced effect on ChE activity. Because of this unusual effect and the shortage of dogs, no ChE₅₀ could be obtained. There were no deaths at these concentrations; the LC₅₀ is obviously greater than 37.13 mg/m³.

Oral

The plasma ChE₅₀ for rats exposed acutely to 0.175 to 7.00 mg/kg ethyl parathion by the oral route is 2.55 (2.12 to 3.1) mg/kg, and the RBC ChE value is 2.58 (2.12 to 3.14) mg/kg. The LD₅₀ for rats given 4.0 to 10.0 mg/kg is 6.85 (6.18 to 7.60) mg/kg.

In rats exposed orally to doses of 0.05, 0.10, or 0.25 mg/kg for 6 weeks, the 0.05 mg/kg is a no-effect dose for ChE activity; the 0.10 mg/kg dose had a moderate effect; and the highest dose produced pronounced effects on both plasma and RBC ChE activity (55% inhibition of RBC and 48% of plasma). Normal levels were regained at 6 weeks after exposure. There were no toxic signs or deaths.

In dogs exposed acutely to doses of parathion (0.50 to 10.0 mg/kg) by the oral route, the plasma ChE₅₀ is 1.67 (0.97 to 2.96) mg/kg and the RBC ChE₅₀ is 1.5 (1.06 to 2.12) mg/kg. The LD₅₀ is calculated to be 8.27 (4.9 to 14.29) mg/kg in dogs exposed to five levels (2.5 to 20.0 mg/kg). Toxic signs (tremors and convulsions) and death were noted at doses above 2.5 mg/kg.

Dogs were given 0.05, 0.10, or 0.50 mg/kg for 6 weeks. Six weeks of exposure to the highest dose, 0.50 mg/kg, inhibited all six test dogs an average of 58% for RBC ChE and 85% for plasma ChE. Normal levels were regained at 6 weeks post dosing. The ChE activity was near normal at 2 weeks post dosing for the 0.05 and 0.10 mg/kg doses. No toxic signs or deaths resulted from these three dose levels of ethyl parathion.

A summary of the subacute cholinesterase responses in the rat and dog are shown in Table 6 and a general summary of the results of the full study are presented in Table 7.

TABLE 6. CHOLINESTERASE RESPONSES IN RATS AND DOGS EXPOSED SUBACUTELY TO ETHYL PARATHION

SPECIES	DOSE MG/M ³	INHALATION EFFECTS ON RBChE	DOSE MG/KG	ORAL EFFECTS ON RBChE
RAT	0.01	NE	0.05	NE
	0.10	ME	0.10	ME
	0.74	PE	0.25	PE
DOG	0.001	NE	0.05	NE
	0.01	ME	0.10	ME
	0.20	PE	0.50	PE

NE < 20% DEPRESSION

ME > 20<40% DEPRESSION

PE > 40% DEPRESSION

TABLE 7. SUMMARY OF RESULTS OF ETHYL PARATHION STUDIES IN RATS AND DOGS

PHASE	PARAMETER	INHALATION		ORAL	
		RAT	(mg/cu m) DOG	RAT	(mg/kg) DOG
ACUTE	RBC CHE ₅₀	5.43	—	2.58	1.50
	PL CHE ₅₀	7.28	—	2.55	1.67
	LC ₅₀	84.0	> 37.0	6.85	8.27
	EC ₅₀ OR ED ₅₀ (TREMORS)	73.7	—	4.48	≈ 9.1
	EC ₅₀ OR ED ₅₀ (CONVULSIONS)	110.6	—	6.69	≈ 10.7
SUBACUTE	NO EFFECT	0.01	0.001	0.05	< 0.05

BEHAVIORAL AND BIOCHEMICAL EFFECTS OF MALATHION^{1, 2}

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The behavioral effects of toxic substances are of interest for several reasons. For one, behavioral tests provide useful information about the effects of chemicals on the central nervous system (CNS). The accumulating literature on the relationship between the behavioral and neurological effects of chronic exposure to methyl mercury illustrates this approach (Evans et al., 1975). However, behavioral tests are not limited to the assessment of central effects. Behavior can also be viewed as the integrated activity of many different systems in the body both in and outside of the CNS, and it follows that a change in behavior can serve as a signal of disorder in any one or more of these systems. Spontaneous motor activity, for example, a very general kind of behavior, may be increased or decreased by changes in muscle tension, body temperature, and blood pressure as well as by changes in neural activity. This kind of general behavioral change can be useful as a flag, telling us that something has happened somewhere in the animal, although it does not provide specific

¹The opinions or assertions contained herein are the private views of the author and are not to be construed as reflecting the views of the Department of the Army or the Department of Defense.

²The experiments reported here were conducted according to the "Guide for Care and Use of Laboratory Animals" (1972) as prepared by the Committee on Revision of the "Guide for Laboratory Animal Facilities and Care" of the Institute of Laboratory Animal Resources, National Research Council.

information as to what exactly is going on. Of course, behavioral tests can and have been designed to examine quite specific effects too, such as changes in color vision (Hanson et al., 1964; Hanson, 1975) or auditory threshold shifts (Stebbins, 1970) after different types of agents.

One of the most important assets of behavioral tests, and one which particularly interests us at the Army Environmental Hygiene Agency, is the sensitivity which behavioral testing procedures frequently provide. Often, we can detect behavioral changes at exposure levels much lower than those found necessary to produce readily observable signs of toxicity. The animals may appear normal after exposure, but their performance in a given behavioral task may betray important changes in the way they perceive or operate in their environment.

I would like to discuss some of the behavioral data we have collected with two pesticide compounds in laboratory rats. I believe both sets of data provide interesting although very different examples of how the sensitivity of behavioral tests can provide useful information about toxicity. First, I'll briefly summarize our findings with the experimental contact insecticide, 4-benzothienyl-N-methylcarbamate, commercially called Mobam®. Second, and in a bit more detail, I'll discuss some of our work with the organophosphate pesticide, malathion, which I believe needs no introduction to this audience.

In studying these compounds, we have used two rather broad behavioral screening tests. We feel these techniques are useful in that they provide sensitive yet economical measures of behavioral change and as I understand it, these are important concerns in the field of toxicology. The first test is the one I mentioned earlier, spontaneous motor activity. The rats are

®Mobam is a registered trademark of Mobil Oil Company, New York, New York. Use of trademarked names does not imply endorsement by the U.S. Army, but is used only to assist in identification of a specific product.

placed into a darkened cylindrical chamber. Infrared photo-beams traverse the inside of the chamber at the level of the grid floor so that when the rat moves about, he trips the photo beams, automatically accumulating activity counts on a mechanical counter. The total count at the end of a five minute test period is taken as the measure of spontaneous motor activity.

The second behavioral test we use provides a general measure of toxic effects on learned behavior, in a paradigm we call "rapid avoidance." Basically, in the jargon of psychologists, it's a one-way avoidance situation, a procedure we modified from one described by Robert Clark some time ago (Clark, 1966). During the test, the animal is placed into a small darkened start box, a tone comes on, and we measure the time it takes for it to enter a larger lighted compartment. If the rat stays in the box for more than 20 seconds, it receives foot shock through the grid floor of the start box until it escapes to the safe side. The beauty of this situation is that it takes only a short time to train the animal to make the avoidance response. In fact, all the training occurs in a single session consisting of six trials the day before exposure. At the beginning of this training session, the animals are exposed to both the shock and tone as soon as they are placed in the start box. Of course, the rats immediately run into the safe compartment. This is repeated in three more trials, each separated by 5-minute intervals. We then give two avoidance trials in which the animals are allowed 20 seconds before the shock comes on. Over 95 percent of the animals we train cross over to the safe compartment in less than 2 seconds on both of these trials. In effect, they've learned the avoidance task, and we've found that we can train five animals in about half an hour in this manner. On the next day, we test animals for their avoidance performance before exposure to the test compound in order to insure that they all retain the avoidance training. Animals showing avoidance latencies over 20 seconds are eliminated. This amounts to about 3 percent of the animals we train; most of the animals, about 90 percent, run to the safe box in less than 2 seconds. So as you can see, we get fairly reliable performance with little time invested in training. Immediately after the preexposure test, the animals receive the test compound and are tested for avoidance in a single trial at a preselected interval following exposure.

In this rather complicated illustration (Figure 1), I've summarized some of the results we have obtained with the carbamate insecticide, Mobam. All the data shown are from rats injected i.p. 15 minutes before test. The dosage range shown here is relatively low, representing only about 1 to 6 percent of the i.p. LD₅₀. The upper portion illustrates the behavioral data and the lower portion the effects of Mobam on brain, plasma, and erythrocyte cholinesterase activity, assessed using a colorimetric method (Gary and Routh, 1965; Levine et al., 1966). We can see a clear dose response function for all measures. It is interesting, though, when looking at the behavioral data at the top part of the figure, that the slope of the avoidance and motor activity functions are really quite different. Avoidance, measured here as the percentage of animals avoiding shock during the test trial, deteriorates rapidly between 2 and 3 mg/kg, while motor activity declines more gradually throughout the dose range explored. Notice particularly the differences we observed at the 2 mg/kg dosage. The avoidance scores of the group given that dose were very similar to those of the control animals given only the corn oil vehicle, but the motor activity of animals given the same dose was significantly depressed, about 70 percent of control group values.

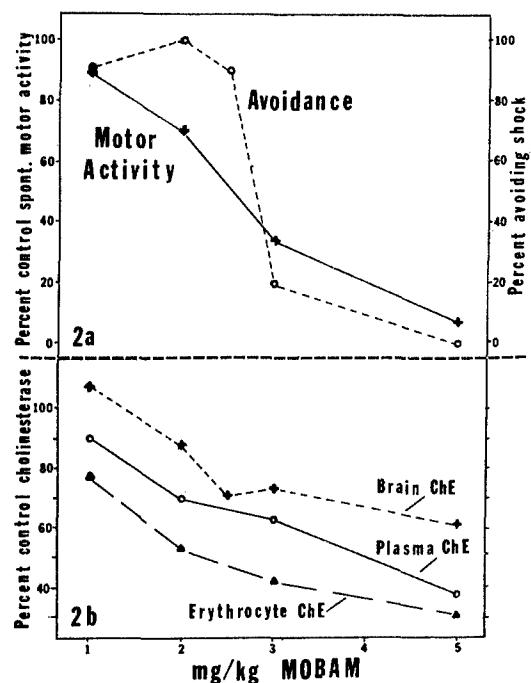


Figure 1. The upper portion shows the percentage of mean control spontaneous motor activity (solid line, left ordinate) and percentage animals successfully avoiding shock (broken line, right ordinate) 15 minutes after injection as a function of Mobam dosage. The lower portion shows the percentage of mean control brain, plasma, and erythrocyte cholinesterase activity 15 minutes after injection as a function of Mobam dosage.

It is also interesting to look at cholinesterase levels after the same treatment. At this dose, 2 mg/kg, we found significant inhibition of plasma and erythrocyte cholinesterase activity, but no significant changes in brain cholinesterase, which was about 90 percent of control levels. With a higher dose, 3 mg/kg, we did find significant brain cholinesterase depression and, as you can see in the upper portion of the figure, a significant, quite dramatic, avoidance impairment. Most of the animals given this dosage did not leave the start box until the shock came on. I should point out that neither of these dosages produced readily apparent signs of toxicity. Independent observers could not tell animals treated with these dosages from untreated controls.

Because the major change in avoidance performance seemed to occur between 2 and 3 mg/kg, we also looked at the effects of an intermediate dosage, 2.5 mg/kg. As the figure shows, most of the animals given that dose successfully avoided shock. However, the percentage score is probably a less sensitive measure of performance than the actual avoidance latencies because the individual avoidance latencies of the 2.5 mg/kg group showed a small but statistically significant increase over those of the control group. The brain cholinesterase levels of this group were also significantly depressed. There appears to be some correspondence, then between the behavioral and biochemical effects of this compound. Doses which produce a significant depression in brain cholinesterase also significantly disrupt avoidance performance. Motor activity is significantly decreased at lower dose levels, dose levels which produce significant cholinesterase inhibition in the blood. It is tempting to speculate that these biochemical and behavioral data may be related. Perhaps central cholinesterase inhibition plays an important part in the effects of this compound on avoidance performance. Our follow-up work with Mobam has been consistent with this interpretation.

Of course, the idea that the behavioral and biochemical effects of cholinesterase inhibitors might be related is not particularly surprising, and it is certainly not new. However, I think it is interesting to observe that a pesticide compound like Mobam may affect behavior in different ways, that some behavioral effects may be more sensitive than others, and that these effects may be mediated by changes in central or peripheral cholinesterase activity to different degrees.

With this background, let me now turn to some of the effects we have found with the organophosphate, malathion. In our first study, we gave rats the avoidance training I described earlier, injected malathion i.p., and then tested avoidance performance 15 minutes, 1 hour, 4 hours, or 24 hours later. Each group consisted of ten male rats, about 300 grams in weight. After the test, we took 1 ml samples of blood by intracardiac puncture. The rats were then decapitated and the brains removed and frozen for cholinesterase analysis later. With this procedure, it was possible for us to obtain behavioral and cholinesterase data from the same animals.

Figure 2 summarizes the effects of malathion on avoidance performance. The dependent measure here is avoidance latency in log seconds. Higher latencies indicate impaired avoidance performance and the principal effect on avoidance appears to occur at the 1 hour postinjection interval. The avoidance latencies of the 50, 100, and 150 mg/kg groups are significantly greater than those of the control group injected with corn oil. No significant effects were observed at the other three postinjection intervals. We observed mild muscle tremor in one of the animals given 150 mg/kg, the highest dosage, 1 hour after injection but the appearance of the other animals was not distinguishable from that of the controls. Thus, with malathion, too, we have observed behavioral changes in animals that appear otherwise normal.

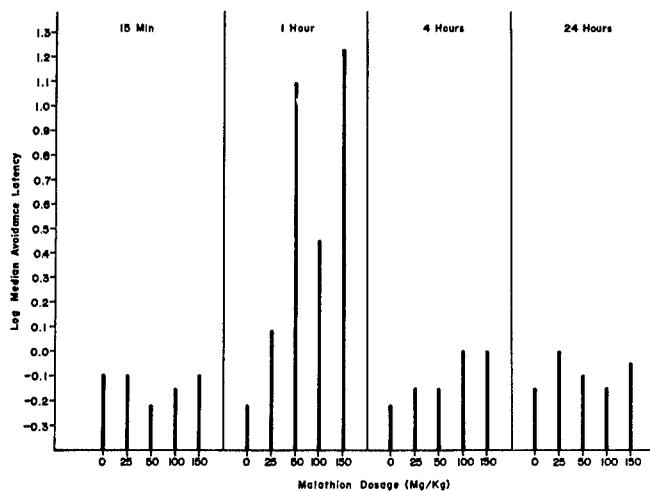


Figure 2. Log median avoidance latencies (seconds) at various intervals following malathion injection. Groups designated 0 mg/kg received the corn oil vehicle only.

You probably notice that the dose response function at 1 hour does not appear as a uniform increase in latency with dosage. The median latency of the 100 mg/kg group is somewhat lower than the next lower and next higher dosages, 50 and 150 mg/kg. While we were originally suspicious that this might simply be due to sampling error or some test artifact, the latency "dip" at midrange dosages has reappeared in subsequent replications of this study, and we now believe that the dose response function with respect to the effects of malathion on avoidance performance may well be bimodal at these low dosages. What biochemical factors might underlie this phenomenon is a matter of further investigation.

The cholinesterase data for the 60-minute injection-test interval groups, the groups in which we found the behavioral changes, are shown in Figure 3. Generally, we have fairly regular dose response functions for all three cholinesterase measures, brain, plasma, and erythrocyte. As with the Mobar data, brain cholinesterase appears least sensitive and erythrocyte most sensitive to the effects of this compound. This is a fairly typical finding. The most important point I'd like to make with this illustration, however, concerns the cholinesterase activity of animals injected with 50 mg/kg. The median avoidance latency of these animals was 12 seconds compared to 1 second in the control group. Yet their cholinesterase levels are 90 percent of control groups or greater. Only with the higher dosage levels do we begin to observe significant cholinesterase depression. Putting the behavioral and biochemical data together, this suggests that low dosages of malathion may disrupt avoidance performance without significantly decreasing cholinesterase activity. In this case, behavioral change appears to be the most sensitive measure of toxicity.

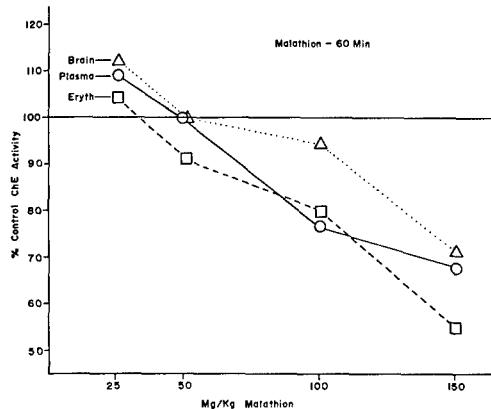


Figure 3. Percentage mean control cholinesterase activity 60 minutes following malathion injection.

The data in Figure 4 depicting cholinesterase activity at an earlier injection-test interval, 15 minutes, suggest further complications. Looking at the 150 mg/kg dose level, we see statistically significant depressions in both erythrocyte and brain cholinesterase activity. Yet, if you remember the behavioral data, this group showed normal avoidance performance. The median avoidance latency of these animals was 1 second, identical to that of the control group. So while in the 60-minute groups we had significant behavioral decrements with normal cholinesterase, now we see significant cholinesterase decrements accompanied by normal behavior. Clearly, with malathion, the behavioral-biochemical relationships are not as neat as they seem to be with Mobam.

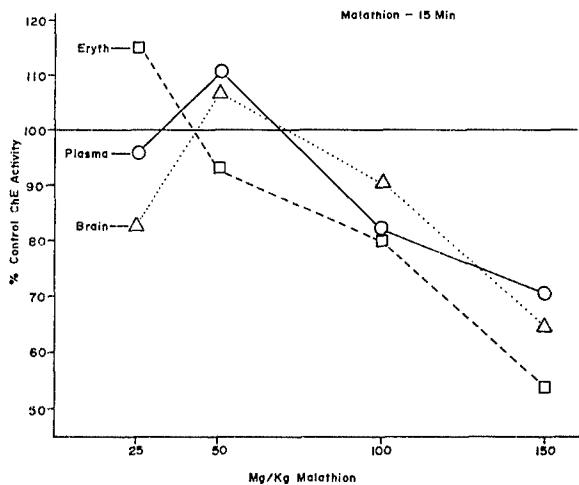


Figure 4. Percentage mean control cholinesterase activity 15 minutes following malathion injection.

The effects of malathion on spontaneous motor activity are also not as clearly defined as the Mobam data. In Figure 5 you see the very sensible dose response function we obtained from Mobam. As I pointed out before, statistically significant decrements in motor activity occurred at 2 mg/kg and higher. The malathion data, in Figure 6, represents a more complex picture. As others have reported, the effects of malathion on motor activity are highly variable. There is a suspicion of a bimodal effect similar to the one that appears in the avoidance data but even with the highest dosage, 150 mg/kg, the apparent depression in motor activity is not

statistically significant. You may remember that with Mobam, we observed motor activity to be a more sensitive test than avoidance; with malathion the opposite appears to be the case. The difference illustrates the importance of employing more than one type of task in the assessment of behavioral toxicity.

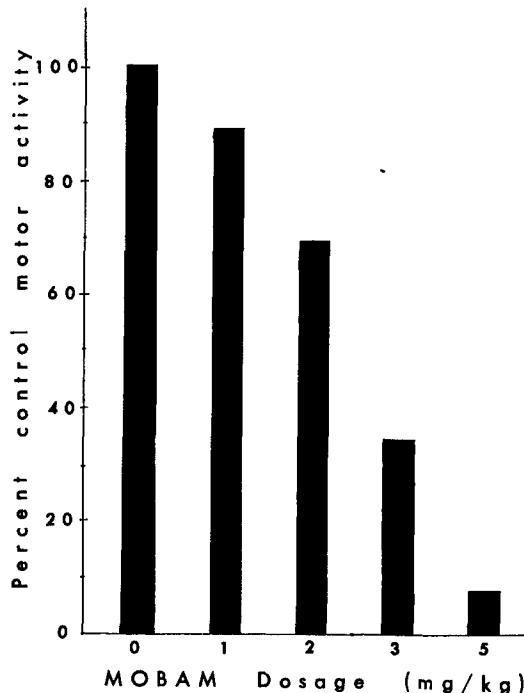


Figure 5. Percentage mean control spontaneous motor activity 15 minutes following Mobam injection.

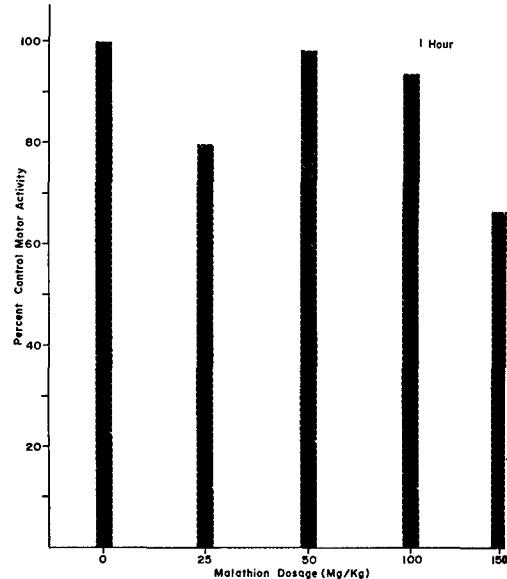


Figure 6. Percentage mean control spontaneous motor activity 60 minutes following malathion injection.

I suppose if I were to summarize these studies with malathion, I would say that significant cholinesterase depression appears to be neither necessary nor sufficient for the appearance of some types of behavioral decrements following malathion exposure in rats. These findings are consistent with behavioral results recently reported after both acute and chronic malathion exposure. Pradhan and Mhatre (1970), for example, reported that rats given 200 and 300 mg/kg subcutaneously showed a significant depression in bar-pressing responses

for water reward although their brain cholinesterase levels were normal. As with our work, malathion effects on spontaneous motor activity were reported to be highly variable but not statistically significant. The results of a 90-day feeding study recently conducted by Dési and his coworkers (1976) also bear on our results. At the end of the feeding period, in which they received 3 or 5 percent of the acute oral LD₅₀ every day, the rats showed normal cholinesterase levels in both blood and brain, yet their EEG patterns were significantly different from normal. It is conceivable that similar changes in EEG may coincide with the behavioral effects we observed. The problem of behavioral or neurological changes without changes in cholinesterase activity is one we believe merits further investigation as it may have some bearing on the adequacy of the measures currently used to monitor human pesticide exposure.

I mentioned earlier that we were particularly interested in the use of behavioral measures as sensitive toxicological tests. I believe the Mobam data illustrate how behavioral tests may help to investigate the interactions between central and peripheral cholinesterase inhibition in pesticide toxicity. The malathion work illustrates how behavioral tests may be sensitive to changes which might otherwise elude our detection. In both cases, I believe, behavioral tests have helped to improve our understanding or at least our appreciation of the possible effects of exposure to low levels of these pesticide compounds.

ACKNOWLEDGEMENT

The technical assistance of Stanley Varnum is gratefully acknowledged. Thanks also to Everett A. Haight and Roger E. Boldt for performing the biochemical analyses. This study was greatly facilitated by the developmental studies conducted by William M. Smith under the direction of Marshall Steinberg.

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TOXICOLOGICAL EVALUATION OF N-HEXYL CARBORANE,
CARBORANYL-METHYLETHYL SULFIDE, AND
CARBORANYLMETHYLPROPYL SULFIDE^{1,2}

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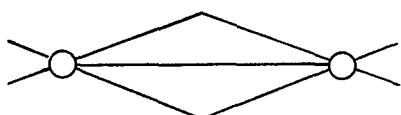
INTRODUCTION

As part of the U.S. Army Environmental Hygiene Agency's mission of support for world-wide health and environmental programs of the Army, the Toxicology Division is frequently called upon to provide information relative to potential health hazards of specific Army operations. In this respect, we operate quite similarly to an industrial toxicology laboratory, without research or development functions. Inherent toxicity and mechanisms of actions of components of military materials or systems are studied only to the extent that they affect the hazards associated with the final formulations to which individuals or groups are exposed. As a result of this, protracted studies are not carried out and frequently the conclusions and recommendations developed raise more questions than they do answers with research-oriented scientists. An example of this situation is seen in the recent toxicological evaluation of three carborane compounds used as reaction stimulators in the development of solid missile fuels.

¹The opinions or assertions contained herein are the private views of the author and are not to be construed as reflecting the views of the Department of the Army or the Department of Defense.

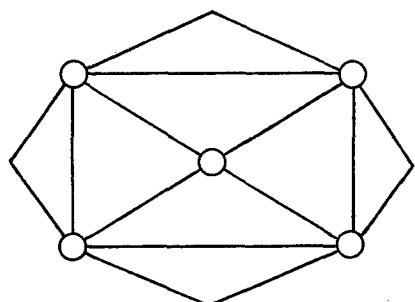
²The experiments reported here were conducted according to the "Guide for Care and Use of Laboratory Animals" (1972) as prepared by the Committee on Revision of the "Guide for Laboratory Animal Facilities and Care" of the Institute of Laboratory Animal Resources, National Research Council.

The number and complexity of known boron hydride derivatives, that is, boranes, has increased phenomenally in the past 30 years, with considerable analytical attention being paid to the internal structural bonding of the polymeric boron hydride compounds. Boron exists as an electron-deficient non-metal in that it has four valence orbitals but only three valence electrons. Therefore, boranes contain a type of bonding not generally observed in other more familiar compounds. Figure 1 shows the idealized structures of diborane and penta-borane. In both of these, we can see a series of shared hydrogen atoms forming B-H-B linkage. The instability of these structures has made them desirable for many industrial purposes, but has also made them severely toxic materials. Boranes have been shown to affect the lungs, cardiovascular system, the central nervous system, skin, kidneys, and liver. Very strict industrial hygiene controls thus are required in all operations involving potential exposure to these materials.



B₂H₆ DIBORANE

Figure 1. B-H-B bonding in borane compounds.



B₅H₉ PENTABORANE

A new class of higher borane derivatives are the carboranes which contain skeletal carbon and boron atoms forming a basket-like structure shown in Figure 2. Incorporation of these carbon atoms into the basket relieves the B-H-B linkage permitting a more stable chemical configuration. However, virtually nothing was known of the toxicological hazards of these materials and military operations employing carboranes have been required to operate under the industrial hygiene controls required for the equivalent borane materials.

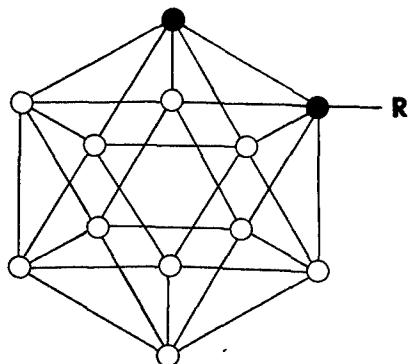


Figure 2. Icosahedral cage structure for orthocarboranes.



CARBORANE

The U.S. Army Missile Command had been using n-hexyl carborane (NHC) and, to a lesser extent, carboranyl methylpropyl sulfide (CMPS) and carboranyl methyl ethyl sulfide (CMES) in solid fuel system preparations working under the industrial hygiene requirements established for equivalent boranes. When the operation was to be expanded to contractor operated facilities, this Agency was asked to provide information on these materials which might permit an alleviation of some of the requirements which had greatly increased the cost and decreased the efficiency of these manufacturing materials. Our request, thus, was to evaluate the potential hazards of these materials within the environment in which they were to be used.

The test chemicals were obtained from the manufacturer, Olin Corporation, Stamford, Connecticut. NHC is a clear liquid with a musty, acrid odor, while CMES and CMPS are slightly cloudy liquids having a pungent sulfide-type odor. The three compounds are soluble in organic solvents such as hexane and acetone but not soluble in water.

SKIN IRRITATION STUDIES

The primary skin irritation potential of the three materials was tested by acute application to the intact and abraded skin of New Zealand white rabbits using the standard tests outlined in the Federal Hazardous Substances Act. All three were found to be primary skin irritants, producing edema and severe erythema, gradually increasing to 72 hours, and leading to the development of eschar formation. Materials were serially diluted in acetone to determine the threshold irritant concentration, using the method of Smythe et al. (1949). In each case, 0.1 percent solutions produced no irritation reaction, applications of 1 percent produced a slight capillary injection in four of five animals at 24 hours, while an application of a 10 percent solution produced similar primary irritant reactions in all test animals at 24 hours. Results of these tests may be seen in Table 1. The hazard for potential sensitization was tested using Hartley strain guinea pigs, with intradermal administration for both test and challenge. None of the compounds produced any recognizable sensitization reaction in guinea pigs and would not be expected to produce sensitization reaction in humans.

TABLE 1. SKIN IRRITATION - RABBITS

	<u>NHC</u>	<u>CMES</u>	<u>CMPS</u>
Primary Irritation	+	+	+
Threshold Irritant Concentration	1%	1%	1%
Sensitization Potential	-	-	-

SYSTEMIC TOXICITY

Systemic toxicity of the three materials was tested in rats, rabbits, and beagle dogs. The results of the rat and rabbit tests are shown on Table 2. In this it can be seen that n-hexyl carborane appeared to be the least toxic when administered orally, intraperitoneally or dermally, although it appeared more toxic when administered intravenously in rabbits. Intravenous administration produced, at lethal doses, lethargy, convulsions, and bloody discharge from the nose. Dermal application of n-hexyl carborane to rabbits produced only severe primary skin irritation with no other toxic manifestations.

Anesthetized beagle dogs were tested to determine the physiological effects of n-hexyl carborane following intravenous administration. Dogs given 50 mg per kg injections of the undiluted material at 30 minute intervals died after two or four injections of material. One dog injected at 30 minute intervals with 25 mg per kg received 8 such injections before dying. In all cases, the heart rate, blood pressure, respiratory rate, and electrocardiogram were recorded. Arterial blood was taken every 30 minutes for blood gas analysis and cell count. Toxic signs developing after repeated applications included bradycardia, lowering of systemic blood pressure, increased respiratory rate (with decreased tidal volume), increase in blood PCO₂ with a decrease in PO₂ and pH. There was no significant change in white or red blood cell count nor in hematocrit or mean cell volume. Necropsy revealed massive pulmonary edema and hemorrhage with capillary destruction.

TABLE 2. SYSTEMIC TOXICITY

<u>Compound</u>	<u>Species</u>	<u>Route</u>	<u>Lethal Dosage (mg/kg)</u>
NHC	Rat	Oral	>9700
		ip	1900
	Rabbit	iv	150
		Dermal	>10000
CMES	Rat	Oral	2085
	Rabbit	iv	320
		Dermal	3890
CMPS	Rabbit	Oral	1900
		iv	320
		Dermal	3160

PRENATAL TOXICITY STUDIES

NHC, CMES, CMPS, sodium salicylate, corn oil and propylene glycol were administered orally to groups of 20 to 30 female rats daily from day 6 through day 16 of gestation and were sacrificed on day 20 by intracardiac injection of sodium pentobarbital. The reproductive tracts were exposed and the corpora lutea, implantation sites and resorption sites were recorded. The fetuses were removed, examined for gross abnormalities and the sex and weight of each fetus was recorded. All grossly abnormal fetuses and 50 percent of the apparently normal fetuses were further studied as Bouin-fixed, freehand sections for soft tissue abnormalities or after alizarin red S staining for skeletal malformations. Results of these tests may be seen in Table 3. All these tests were performed at lethal or near lethal levels; thus, decrease in fetal weight could well be attributed to toxic maternal effects. Only with CMPS treatment was there seen an increase in the number of fetal resorptions. Except for sodium salicylate tested rats, no fetal abnormalities were recorded in any of these animals.

TABLE 3. PRENATAL TOXICITY, RATS (ORAL ADMINISTRATION)

<u>Compound</u>	<u>Dose (mg/kg)</u>	<u>Maternal Deaths</u>	<u>Fetal Resorption</u>	<u>Fetal Weight</u>	<u>Fetal Abnor- malities</u>
NHC	1000	0	No difference	Decreased	0
CMPS	190	17/30	Increased	Decreased	0
CMES	120	13/22	No difference	Decreased	0
Sodium Salicylate	200	2/30	No difference	Decreased	+
Corn Oil	1000	0	-	-	0

All three compounds were submitted for in vitro mutagenic evaluation using an Ames-type bacterial/yeast system. Neither NHC nor CMPS was reactive either by direct exposure or when activated through incubation with tissue homogenates of liver, lung, and testes from rat, mouse or monkey. CMES, however, when tested against salmonella typhinurium TA-1535, produced a consistent, weak response after activation by incubation with the liver tissues of rats, mice and monkeys.

INHALATION TESTING

Groups of six male rats each were exposed to saturated vapors of NHC, CMES, and CMPS for eight hours. In one test, the vapors were generated at room temperature and in a separate test, bubblers were maintained at a temperature of 65 C. The results of these tests can be seen in Table 4. Although the saturated vapor levels are quite low, eight-hour exposures were capable of producing gradually developing pulmonary abnormalities at least in part of the animals. However, the temperature necessary to generate these levels was significantly in excess of that anticipated in manufacturing operations.

TABLE 4. ACUTE INHALATION TOXICITY - RATS

<u>Test</u>	<u>Compound</u>	<u>Conc. (mg/l)</u>	<u>Abnormal Effects</u>
Saturated Vapor 8 Hours at 24 C 6/Group	NHC	0.0	None
	CMES	0.1	None
	CMPS	0.1	None
Saturated Vapor 8 Hours at 65 C 6/Group	NHC	0.1	Emphysema in 3 of 6 rats
	CMES	0.5	Emphysema in 5 of 6 rats
	CMPS	0.4	No lesions or changes

Groups of rats were also exposed to graded aerosol concentrations for one or four hours. Results of these tests are shown in Table 5. There we can see that exposure for longer than one hour produces an exacerbation of minimal pulmonary infections existing within the colony.

TABLE 5. ACUTE INHALATION TOXICITY - RATS

<u>Test</u>	<u>Compound</u>	<u>Conc. (mg/l)</u>	<u>Abnormal Effects</u>
Aerosol 1 hr Exposure 10/Group	NHC	2.0	No lesions or changes
	CMES	2.1	No lesions or changes
	CMPS	2.1	No lesions or changes
Aerosol 4 hr Exposure	NHC	1.4	No lesions or changes
	CMES	0.9	Pneumonitis in 3 of 10
	CMPS	0.9	Pneumonitis in 4 of 10
	NHC	3.2	No lesions or changes
	CMES	1.9	Pneumonitis in 3 of 10
	CMPS	1.5	Pneumonitis in 3 of 10

Subchronic inhalation hazard for NHC was tested by exposing male beagle dogs and male albino rats to either 77 mg per cubic meter or 245 mg per cubic meter for six hours a day, five days a week for six weeks. Chamber air samples for both concentrations were analyzed by gas chromatography. All animals used in these experiments were observed during a preliminary period and control groups exposed to chamber air were matched with each treatment group in respect to number, age, sex and body weight. Animals were weighed weekly and observed daily for general appearance and behavior. Periodic clinical and hematological examinations were made to establish baselines on dogs for preexposure control values and during the course of the experimental period. Dogs were sacrificed and necropsied at the end of six weeks while ten rats were necropsied at three weeks and ten at six weeks after the beginning of the exposure period. Groups of ten exposed and ten control rats were held for four weeks postexposure before necropsy. The following organs were removed at necropsy and processed for histopathological examination: brain, pituitary, eyes, nasal turbinates, thyroid, heart, trachea, esophagus, stomach, small and large intestines, pancreas, liver, kidneys, adrenals, testes, prostate, skeletal muscle, and bone.

Dogs and rats exposed to either concentration showed gasping and excessive preening during each exposure in the first week (Table 6). These signs disappeared overnight. No clinically significant changes were found in any of the blood parameters measured in dogs. No significant organ to body weight changes

between test and control rats were noted. No chemically induced gross or histopathological lesions were observed except in the lung tissue sections of dogs. Lungs of NHC-exposed dogs showed areas of interstitial pneumonitis, indicative of developing emphysema. Control dog lungs were clear. This indicated again that repeated or prolonged exposures to aerosols of NHC may produce transitory respiratory irritation which may result in progressive pulmonary damage.

TABLE 6. SUBCHRONIC INHALATION - RATS AND DOGS

<u>Test</u>	<u>NHC</u>	<u>Concentration (mg/m³)</u>	<u>Abnormal Effects</u>
Aerosol, 6 hours per day, 5 days per week for 6 weeks		77 245	Gasping and preening during exposure for first week Interstitial pneumonitis in dogs at necropsy (six weeks after exposure)

NOTE: Concurrently exposed animals were studied for prenatal toxicity and operant behavioral changes induced by exposure. Neither effects were observed.

DISCUSSION

As a result of these tests, it was evident that the technical grade NHC, CMES and CMPS should be handled with caution, using skin and eye protective equipment. Any of the compounds coming into contact with unprotected skin or eyes should be removed immediately. At the present time, good industrial practices would indicate that mechanical control of vapors and aerosols of the carborane compounds in the working areas would be necessary to contain and prevent possible exposure hazards to man. While environmental levels have not been established for airborne concentrations of NHC, the most likely processes requiring control are those in which fogs or mists are involved or where hot solutions of NHC are employed.

These tests indicate that with appropriate safety precautions, technical grade NHC, CMES, and CMPS should present little acute toxic hazard to man. NHC appears to be the least toxic of the three compounds studied. NHC and CMPS appear to be nonmutagenic on the basis of microbial assays, while CMES appears to be weakly mutagenic.

As a result of these tests, the processes in manufacture of NHC and CMPS could be engineered in a manner to eliminate many of the constrictions related to borane hazards. Because of the potential mutagenic changes revealed in this in vitro test situation, CMES was withdrawn from the process line as a carborane for use in formulation of solid fuel systems.

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HAZARD EVALUATION OF MUNITION COMPOUNDS

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and
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The US Army Medical Research and Development Command is supporting research to determine the human health and environmental hazards associated with the Army's conventional munitions industry.

The specific objective of this research program is to assemble the scientific data-base for munitions unique water and air pollutants from which environmental quality standards can be established.

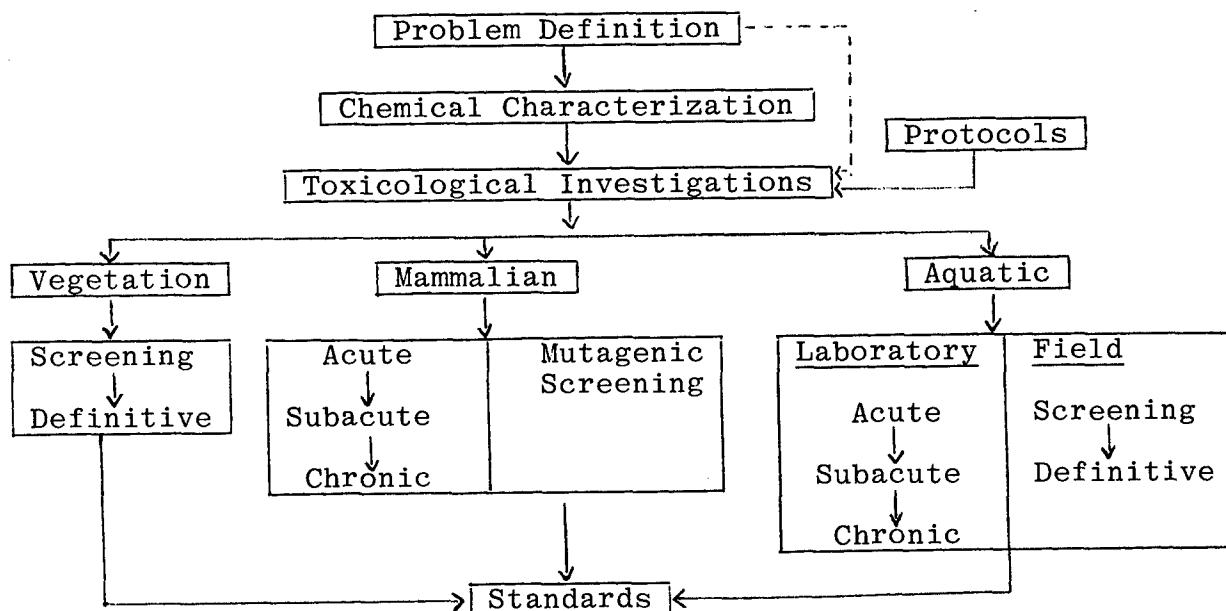
Environmental Quality Standards are required to allow compliance with present and pending environmental legislation. The Federal Water Pollution Control Act as amended in 1972, the Clean Air Act as amended in 1974, and the National Environmental Policy Act all provide requirements to limit discharge of pollutants and to assess their impact in the environment. In addition, considerable regulatory authority is delegated to the States and other local agencies who may establish additional pollutant discharge limitations.

The Surgeon General of the Army has undertaken responsibility for environmental health standards research for Army-unique pollutants. The responsibility of establishing environmental quality standards lies principally with the US Environmental Protection Agency, but also with several other Federal, State, and local agencies. EPA's development of criteria for munition standards, however, will not be timely to impact the Army's Munitions Production Base Modernization and Expansion Program. In addition, at the

local level individual Army Ammunition Plants are faced with increasingly stringent discharge limitations thru the issuance of National Pollutant Discharge Elimination System Permits. Thus, it is urgent that the Army develop criteria for environmental quality standards. When sufficient data become available they are presented to the EPA and other regulatory agencies for review, interpretation, and publication of Federal environmental quality standards. This requires close coordination of the Army's research program with the regulatory agencies and the scientific community to insure the technical excellence and credibility of the research data. One method used to achieve this goal is by periodic review of the research by the National Academy of Sciences Committee on Military Environmental Research.

Accepted environmental quality standards will have an impact on all aspects of the Army's munition industry. These include production, load assembly and pack, and demilitarization; at both existing facilities (operating standards) and planned facilities (design standards, environmental impact statements). Since many of the contaminations are of concern in both the industrial and general environment, the requirements for proper industrial hygiene practices and pollution abatement systems can be addressed concurrently. Scientifically based standards will provide the necessary justification for the development and implementation of cost-effective control measures that ensure the protection of human health and the environment.

The research approach for this program is outlined in the following schematic diagram:



In the case of air pollutants arising from munitions manufacturing processes we will, of course, be concerned with the vegetation and mammalian pathways. For water pollutants, mammalian and aquatic toxicity studies are of concern.

From the mammalian, aquatic and vegetation toxicology studies, extrapolation of the data can be made following the rationale set out in Table 1. Safety factors are introduced depending on the status of available data.

TABLE 1. DATA EXTRAPOLATION RATIONALE

Mammalian Studies:

Acute	Subacute	Chronic
No Extrapolation	1/100 of	1/100 of
Possible	No Effect	No Effect Level
	Level	(Carcinogens require special interpretation)

Aquatic Lab Studies:

Acute	Subacute	Chronic
From 1/10 to 1/100 of Mean		No Effect
Lethal Concentration		Concentration
		Used Directly

Aquatic Field Studies:

Screening	Definition
Data used for verification of lab studies	
Data and indication of synergistic activity	

Because most of our toxicology studies are still incomplete we have only determined temporary guidelines. No final guidelines or standards have been set for any of the munition compounds. Temporary guidelines that have been determined in six different classes of munition pollutants will be outlined.

Firstly, the different categories are given in Table 2 with the numbers of the toxic compounds involved in each area. Of course, the individual component of a pollutant mixture showing the greatest toxicity will be used to derive the standard or guideline for that mixture. For example, for TNT production wastewater the principal component, 2,4-DNT, is also the most toxic in mammalian studies conducted to date.

TABLE 2. CATEGORIES OF MUNITIONS POLLUTANTS

	Number of Compounds	
	<u>Water</u>	<u>Air</u>
1. TNT Production	20	4
2. TNT Load Assembly & Pack	11	0
3. RDX/HMX Production	8	3
4. Nitroglycerine	6	0
5. Nitrocellulose	1	0
6. White Phosphorus Load Assembly & Pack	1	0

Secondly, the criteria used for setting the guidelines in the six categories are detailed in Tables 3 to 8. Two guidelines are provided for TNT related wastewaters due to the significant chemical differences of wastewater from load, assembly and pack (LAP) operations and those from TNT production (See Table 3,4).

TABLE 3. TNT LOAD, ASSEMBLY & PACK WASTEWATER

Temporary Guideline: 0.05 ppm as TNT

Criteria:

Criteria:	<u>No Effect Level</u>	<u>Basis</u>	<u>Certainty</u>
Mammalian	No Estimate	Partial Acute	--
Aquatic Lab	0.05 ppm x 50	Partial Acute	Low
Aquatic Field	0.01 to 0.3 ppm	Partial Definitive	Low

TABLE 4. TNT PRODUCTION WASTEWATER

Temporary Guideline: 0.025 ppm as 2,4-DNT

Criteria:

<u>Criteria:</u>	<u>No Effect Level</u>	<u>Basis</u>	<u>Certainty</u>
Mammalian	0.025 x 1000	Partial Chronic	Medium
Aquatic Lab	No Estimate	Partial Acute	--
Aquatic Field	0.03 to 0.3 ppm	Partial Definitive	Low

The criteria for setting the interim guidelines for both RDX/HMX and nitroglycerin wastewaters are set out in Tables 5 and 6.

TABLE 5. RDX & HMX PRODUCTION WASTEWATER

Temporary Guideline: 0.25 ppm as RDX

Criteria:

<u>Criteria:</u>	<u>No Effect Level</u>	<u>Basis</u>	<u>Certainty</u>
Mammalian	0.25 ppm x 100	Partial Chronic	Medium
Aquatic Lab	0.5 ppm	Partial Chronic	High
Aquatic Field	0.02	Screening	Low

TABLE 6. NITROGLYCERINE WASTEWATERS

Temporary Guideline: 0.04 ppm as nitroglycerine

Criteria:

<u>Criteria:</u>	<u>No Effect Level</u>	<u>Basis</u>	<u>Certainty</u>
Mammalian	0.18 ppm x 1000	Subacute	High
Aquatic Lab	0.04 ppm	Partial Chronic	High
Aquatic Field	No Estimate	Partial Screening	--

No guideline for nitrocellulose wastewaters has been established since nitrocellulose has not been found to cause toxic effects at concentrations up to 100 ppm in mammalian species and 1000 ppm in aquatic organisms (See Table 7).

TABLE 7. NITROCELLULOSE WASTEWATER

Temporary Guideline: None Related to Toxicity - Nutrients and Suspended Solids will be Controlling Factors.

Criteria:

<u>Criteria:</u>	<u>No Effect Level</u>	<u>Basis</u>	<u>Certainty</u>
Mammalian	Greater than 100 ppm	Subacute	High
Aquatic Lab	Greater than 1000 ppm	Acute	High
Aquatic Field	Greater than 20 ppm	Partial Definitive	Low

For white phosphorus an extremely low temporary guideline has been recommended. It should be noted that this extremely toxic material is of concern at only one location - Pine Bluff Arsenal, Arkansas (See Table 8).

TABLE 8. WHITE PHOSPHORUS WASTEWATER

Interim Guideline: 0.01 ppb as White Phosphorus

Criteria:

<u>Criteria:</u>	<u>No Effect Level</u>	<u>Basis</u>	<u>Certainty</u>
Mammalian	0.1 ppm x 1000	Acute	Low
Aquatic Lab	0.6 ppb	Partial Chronic	High
Aquatic Field	0.01 ppb	Definitive	High

These temporary guidelines are the first products of this munitions research program. Updates of these and/or recommendations of final standards will be accomplished annually. Estimates of the completion dates for the six wastewater classes for which temporary guidelines have been developed are set out in Table 9.

TABLE 9. MUNITIONS STANDARDS RESEARCH COMPLETION DATES

<u>Wastewater</u>	<u>Date</u>
TNT-Production	September 79
TNT-LAP	September 79
RDX/HMX	April 77
Nitroglycerine	February 78
Nitrocellulose	December 77
White Phosphorus	December 76

Forecasts of the completion dates for other munitions-related air and water pollutants must await the completion of problem definition studies and, in most cases, the generation of initial and then the complete toxicological data profile.

AMRL-TR-76-125

PAPER NO. 15

TOXICOLOGICAL PROGRAMS OF THE US ARMY MEDICAL BIOENGINEERING
RESEARCH AND DEVELOPMENT LABORATORY - AN OVERVIEW

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The US Army Medical Bioengineering Research & Development Laboratory of the Medical Research & Development Command was re-organized in 1972 and given an additional mission to investigate the Army's environmental problems, in particular those problems relating to water, air and soil pollution as they affect both human community health and the health of the soldier.

The Environmental Protection Research Division was established to undertake this job and presently has a staff of about 50 and an annual spending budget of almost \$7 million dollars.

The Laboratory is undertaking research and development in the 10 areas which are listed below.

1. Munitions Standards
2. Land Application of Wastewater
3. Wastewater Reuse
4. Water and Wastewater Treatment
5. Analytical Methods
6. Pesticide Disposal
7. Industrial Hygiene/Occupational Health
8. Technical Information System/or Studies
9. Installation Restoration Standards
10. Basic Research.

I propose to outline for you the extensive toxicological programs that we have developed and implemented for obtaining data bases and for the ultimate establishment of environmental quality standards.

The programs also impinge on the water and wastewater areas and industrial hygiene/occupational health. The Laboratory has an in-house research capability covering basic and analytical research, microbiological research, and aquatic toxicity research. The specific objective of this research program is to assemble the scientific data base from which environmental quality standards can be established. Interim standards, or guidelines as we prefer to call them at this stage, have been set for some of the Army's munitions compound and the rationale for this was presented in the preceding paper.

The objectives of Standard Development are as follows:

1. To provide water quality guidelines for safe drinking water, recreation, irrigation, and protection of aquatic life.
2. To provide guidelines for protection of wildlife and domestic animals.
3. To provide safe dietary levels for humans.
4. To provide water treatment target levels for water leaving Army arsenals and bases.
5. To provide restoration standards for land intended for release (public use).
6. To provide standards for protection of workers engaged in land restoration and water restoration.

With regard to the last two objectives, we have initiated under the installation/restoration program, an evaluation of the toxic potential of soil pollutant chemicals. These are both organic and inorganic chemicals used for a variety of purposes that have been manufactured, tested, stored and disposed of in relatively high concentrations in small isolated areas by the Army, Navy and Air Force.

From the data bases of some 11 chemicals the appropriate numbers have been extracted and Soil Pollutant Limit Values's (SPLV's) calculated.

The compounds are:

- | | |
|----------------------------------|--------------------------|
| 1. Mercury and Mercury Salts | 7. Methylphosphonic Acid |
| 2. Arsenic | 8. Aldrin |
| 3. Chlorate Salts | 9. Dieldrin |
| 4. Dicyclopentadiene | 10. Endrin |
| 5. Isopropyl Methylphosphonate | 11. Chlordane. |
| 6. Diisopropyl Methylphosphonate | |

This new concept of SPLV's is an estimate that will be compatible with the protection of man in the soil environment just as a TLV is an estimated value for the protection of man in the working exposure environment.

A preliminary communication describing the SPLV concept was made last March at the annual Society of Toxicology Meeting (see Toxicol. Appl. Pharmacol., (1976), 37:104).

The approach to standards development is as follows - problem definition studies are first undertaken followed by development of research protocols. Chemistry and toxicology studies are initiated usually by the contract procedure, and when completed, interpretation and recommendations for standards can be made.

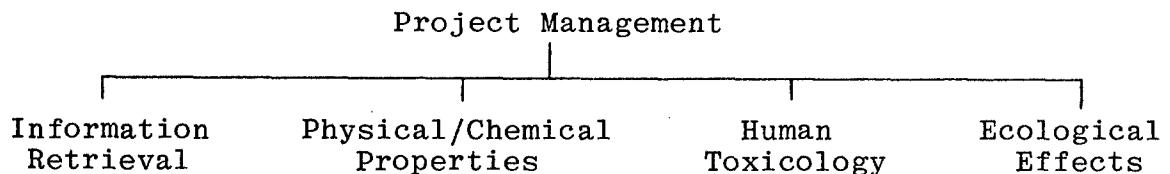
The Problem Definition Study has as its objectives the following:

1. To provide a data base of physical, chemical and biological properties for each pollutant.
2. To provide information on analytical methods that can be used in toxicological studies and in the OTSG sample analysis program.
3. To identify health and environmental hazards associated with each pollutant as it exists at an installation.
4. To calculate pollutant limit values which may be used as preliminary target levels to assess potential restoration processes for land use modeling.
5. To identify areas and priorities where research is required to develop an adequate base for the assessment of adverse environmental effects.

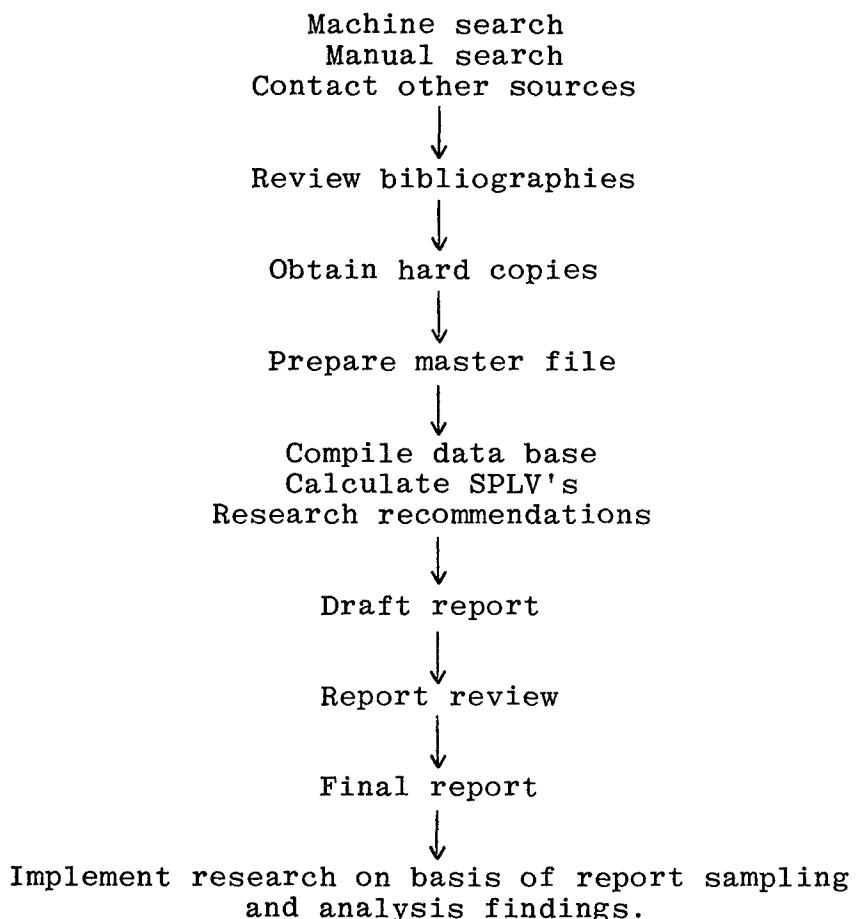
For problem definition studies we assemble an interdisciplinary team (in-house personnel plus consultants) with the expertise in the following areas:

1. Chemistry
2. Chemical and Environmental Engineering
3. Toxicology (+ Inhalation)
4. Aquatic Toxicology and Microbiology
5. Ecology
6. Entomology
7. Technical Information
8. Plant Physiology
9. Soils Chemistry.

This team under the Project Manager is divided into the following 4 areas.



In detail the problem definition study is set out as follows:



For those interested in our problem definition studies - 11 reports have so far been published as Technical Reports of the Laboratory and they cover some 13 munitions, 6 or more airborne pollutants, and 22 other Army Environmental pollutants.

The details of our toxicological protocols are set out in Tables 1-4. We define the tasks in four phases and each phase must be completed before proceeding to the next phase. The approximate time requirements are shown on each protocol.

TABLE 1. MAMMALIAN TOXICOLOGICAL PROTOCOL

<u>Time (Mos.)</u>	<u>Task</u>
6	I. ACUTE TOXICITY: SINGLE EXPOSURE Acute LD ₅₀ 's, ocular and dermal studies, preliminary metabolism and mutagenic screening.
6	II. SUBACUTE TOXICITY: MULTIPLE EXPOSURE 90-day feeding study, detailed metabolism, preliminary carcinogenic, mutagenic and teratogenic evaluations.
24+	III. CHRONIC TOXICITY: LIFE-TIME EXPOSURE Life-time exposure and reproduction study with detailed observations for carcinogenic, teratogenic and mutagenic effects.
6-24	IV. MULTIPLE COMPOUND TOXICITY Evaluate synergistic and antagonistic effects.

TABLE 2. WILDLIFE TOXICOLOGICAL PROTOCOL

<u>Time (Mos.)</u>	<u>Task</u>
3	I. ACUTE TOXICITY: SINGLE EXPOSURE Determine LD ₅₀ , ocular and dermal studies, preliminary metabolism.
3	II. SUBACUTE TOXICITY: MULTIPLE EXPOSURE Determine LD ₅₀ following 5-10 days dosing, observation of physiological effects.
6-24	III. CHRONIC TOXICITY: LONG-TERM EXPOSURE Determine no-effect level following long-term feeding study (min. 20 days to $\frac{1}{2}$ species life span), evaluate physiological, reproductive, and teratogenic effects.
3-12	IV. MULTIPLE COMPOUND TOXICITY Evaluate synergistic and antagonistic effects.

TABLE 3. AQUATIC TOXICOLOGICAL PROTOCOL

<u>Time (Mos.)</u>	<u>Task</u>
6	I. STATIC BIOASSAYS 96 Hr LC ₅₀ 's (fish), 48 Hr LC ₅₀ 's (inv.), 96 Hr EC's (algae), influence of temperature, pH, hardness, aging, and life stages, preliminary bioaccumulation.
6	II. DYNAMIC BIOASSAYS Incipient bioassays of mean lethal concentration, define influence of environmental factors and bioaccumulation ratios.
12	III. CHRONIC BIOASSAYS Life-time exposures to define no effect concentration, observations of growth, reproduction, and physiological effects.
3-6	IV. MULTIPLE COMPOUND TESTING Evaluate synergistic and antagonistic effects on toxicity or bioaccumulation.

TABLE 4. VEGETATION PHYTOTOXICITY PROTOCOL

<u>Time (Mos.)</u>	<u>Task</u>
Cont.	I. SOIL AND WATER SCREENING Short-term evaluation of phytotoxic properties of contaminated area soil and water samples.
6	II. COMPOUND SCREENING Determination of approximate dose range for effects and potential for bioaccumulation.
12	III. DEFINITIVE COMPOUND TESTING Determination of dose-response curves and bioaccumulation ratios, observations of sublethal effects on growth, yield, and seed germination.
6-12	IV. MULTIPLE COMPOUND TESTING Evaluate synergistic or antagonistic effects on phytotoxicity or bioaccumulation.

Many toxicological studies - chemical, mammalian, aquatic, wildlife and vegetative - are in progress and the majority of the organizations that are doing this work are listed below:

1. Chemical characterization of pollutants:

Edgewood Arsenal Chemical Labs.
Natick Labs.
Picatinny Arsenal
Naval Surface Weapons Center
Holston Army Ammunition Plant
Midwest Research Institute
Stanford Research Institute

2. Mammalian toxicology:

U.S. Air Force - Aerospace Medical Research Labs.
Office of Naval Research
Midwest Research Institute
Stanford Research Institute

3. Aquatic toxicology:

Bionomics, Inc.
Water & Air Research, Inc.
Battelle Columbus Labs.
Environmental Control Technology Corp.
Stanford Research Institute
Edgewood Arsenal - Ecology Research Labs.

As already mentioned, interim guidelines for some of the munition compounds have already been set. These values have been derived from data already available from mammalian, aquatic and vegetative studies. As the data from other areas - domestic animals, wildlife - become available, final guidelines and standards will be recommended.

Table 5 lists the munition compounds that are being investigated in our present research program.

Current research on air pollutants of concern is presently being carried out on the following compounds: methyl nitrate, tetrinitromethane, nitromethane and the mononitrotoluenes.

TABLE 5. PRESENT RESEARCH ON DISCHARGES AND EMISSIONS RELATED TO THE FOLLOWING PRODUCTS

TNT PRODUCTION - Condensate Water

2,4-DNT; 2,6-DNT; 2,3-DNT; 2,5-DNT; 3,4-DNT; 3,5-DNT; Tetranitromethane (air pollutant); Mononitrotoluene (air pollutant).

TNT LOAD ASSEMBLY AND PACK - Pink Water

2,4,6-TNT; 1,3,5-Trinitrobenzene (TNB); 2,4,6-TNB Aldehyde; 2,4,6-TNB Alcohol; 2,4,6-TNB Nitrile; 2,4,6-TNB Aldoxime; 1,3-Dinitrobenzoate; 4,6-Dinitroanthranilic acid; 3,5-Dinitrophenol; 4-Amino 2,6-DNT; 6-Amino 2,4-DNT; many other compounds in trace quantities.

RDX/HMX - Methyl Nitrate (air pollutant)

NITROGLYCERINE - Trinitroglycerine; 1,2-Dinitroglycerol (DNG); 1,3-DNG; 1-Mononitroglycerol (MNG); 2-MNG; Nitrodiphenylamine.

NITROCELLULOSE

WHITE PHOSPHORUS - Elemental Phosphorus (P₄)

PRIMING COMPOUNDS - Tetrazene; Lead Styphnate; Trinitroresorcinol; Pentaerythritol Tetranitrate.

Further toxicological studies will shortly be initiated on many other Army unique chemicals such as the components of colored smokes and pyrotechnics, and the impurities and degradation products of the many toxic agents and munitions that have been disposed of or dumped at Army arsenals for very many years. Some of these munitions pollutants are listed in Table 6.

TABLE 6. MUNITIONS POLLUTANTS - RECENT INITIATIONS

NITROGUANIDINE

PROPELLANTS:

Diphenylamine
Dibutyl Phthalate

TRACERS:

Red Phosphorus
Potassium Perchlorate

PYROTECHNICS:

Benzanthrone
1,4-Di-p-toluidinoanthraquinone
1,4-Diamino-2,3-dihydroanthraquinone
1-Methylaminoanthraquinone
Dibenz(b,def)chrysene-7,14-dione
Hexachloroethane
Hexachlorobenzene

OPEN FORUM

DR. FRIESS (Naval Medical Research Institute): I would like to start with a question for Mr. Owens about ethyl parathion. There seems to be an increasing concern with organo phosphorus insecticides about the problems of delayed neuropathy or neurotoxicity. I wonder in this connection whether you held any of the dogs that you have had on subacute exposure, particularly the high dose dogs that had tremors and convulsions. Did you note the incidence of any delayed neuropathy, any paralysis after some lag time of the order of 10 to 50 days in your dogs that had multiple exposures?

MR. OWENS (Edgewood Arsenal): These dogs that we used were held for 6 months postexposure and exhibited apparent complete recovery. We saw no latent effects of the exposure to ethyl parathion.

DR. FRIESS: No weakness of the hind limbs and no temporary paralysis?

MR. OWENS: No, sir.

MR. VERNOT (University of California, Irvine): My question is for Dr. Dacre. Your aquatic limit for white phosphorus was 0.01 ppb, and I wondered how you measured this. Is there an analytical technique that will allow you to detect that low level of phosphorus in water?

DR. DACRE (U.S. Army Medical Bioengineering Research and Development Laboratory): White phosphorus is highly toxic to fish. We have measured that concentration in fish.

MR. VERNOT: So it's a biological method for fish?

DR. DACRE: Yes.

DR. MURPHY (Harvard School of Public Health): This was based upon a no-effect level at 0.025 ppm which was something that you called partially chronic and with the added comment that this was highly mutagenic. And I wonder how you arrived at 0.025 ppm as a temporary guideline with that kind of basis.

DR. DACRE: The compound selected was 2,4-dinitrotoluene. Two-year chronic studies have not been completed on it. The data we used for that was the 90-day study which had been completed in rats and mice. I think the rat datum was selected. And that would be the no-effect level that we found in rats and in that case a safety factor of 100 was introduced to give that figure.

DR. MURPHY: A safety factor of 100 was used?

DR. DACRE: Yes.

DR. MURPHY: Well, I misinterpreted the slide then because it showed 0.026 ppm as a no-effect level and then showed .025 ppm as the guideline.

DR. DACRE: If 0.025 ppm is the interim guideline, the no-effect level would be 2.5 ppm.

DR. MURPHY: That was not what was on the slide.

DR. DACRE: I'm sorry.

DR. FRIESS: Dr. Dacre, could I continue that questioning for just a moment because it's a matter of increasing national interest to have a philosophy by which safety factors for particular kinds of chemicals are applied in a uniform way throughout this national structure which you have been working in. And I've not been able to find anywhere a philosophy document or a guideline which sets up some rationale for kinds of safety factors applied to no-effect levels in sensitive species to lead to temporary guidelines or even to more permanent guidelines. To your knowledge, is there in existence, within the Army or elsewhere, a philosophy document which sets up classifications of safety factors related to chemical compound class?

DR. DACRE: The original concept of extrapolating data from animals to man, of course, was developed in Dr. Lehman's laboratory and that was published in that classical book on appraisal of the safety of food additives published by the association of the official Food and Drug chemists. The concept of applying safety factors is not well defined. It's largely a matter of a certain amount of guesswork. But it has been looked at very, very closely by the World Health Organization. As you know,

they use a very similar concept for establishing ADI's, that's "Acceptable Data Intakes," for food additives and pesticide residues. The safety factor selected depends on the type of test, the type of animal, and things like that.

DR. FRIESS: It's just at that point that the obscurity sets in. The safety factor seems to depend in part on the conservatism of the leader of the panel which happens to consider a particular chemical. Now, I wonder if we can somehow move from there to establish at least a set of rational tentative proposals for kinds of safety factors for particular classes of compounds.

DR. DACRE: We probably will have to. We have just had discussions on setting an interim guideline for 2 compounds which are of concern to the Army because the Army is involved in litigation involving these 2 compounds. They are compounds escaping from an Army arsenal that are affecting farmers' cows and crops adjacent to the arsenal. They are not highly toxic in their own right but they have some mutagenic activity which we are trying to quantify. In these 2 instances, we've set a safety factor of 1000.

DR. FRIESS: Is there any chance, do you think, that EPA might be gutsy enough at this stage of its existence to begin the promulgation of a philosophy of safety factors ranging from 1000 down to 10 and related to compound class or principal toxic effect?

DR. DACRE: I think they'll have to do that. All the data that we derived has already been sent to them for their comments and for their criticism. We found it very difficult to get any sort of concrete reply. In fact, we find it difficult to get any sort of reply out of EPA at all.

DR. BACK (6570 Aerospace Medical Research Laboratory): This concept of setting standards for standard safety factors bothers me quite a bit because as I've reviewed 2 to 4 criteria documents per month for the past year and a half, I see there is absolutely no correlation between the data and the safety factor inherent in the proposed standard. And I don't think there is any way of assessing an absolute figure for this kind of numbers game. If you're setting a long term standard for a working population as

opposed to a standard for a high level exposure for short periods of time, there is a great deal of difference in how it should be done, one of them based upon the steepness of the dose response curve which is a reflection of the manner in which the compound exerts its toxicity. If a compound has a long low slope of activity then that means that as you go from a very low dose to a very high dose, you have very poor reproducibility which probably means that the compound isn't working in a single target organ but working in many places depending upon the age of the animal or the age of the man. That may also mean that at high dose levels, it's working on the kidneys and liver and the heart; and at low levels, it's working on the hematopoietic system or some other system. So I don't think there is any way of setting and assessing the way in which you are going to set a safety factor. And I hope at this stage of the game that EPA doesn't set rules for safety factors because again, we are playing with a numbers game and it will be all black and all white. And I don't like to see all black and all white when one makes a judgement because a judgement must be based upon the compound, not a class of compounds. As you well know, the change from an alpha form to a beta form in naphthylamine makes one a mutagen and one not a mutagen. The change in one methyl group makes a compound, especially the hydrazines, a hepatotoxin as opposed to a nonhepatotoxin. So putting compounds in categories and saying we'll put a safety factor of 10 on one and 100 on another and 1000 on another just doesn't work out.

DR. HENDERSON (Olin Corporation): If I recall correctly, Dr. Dacre, you said that you had set soil pollutant limit values for mercury and its salt. Could you expand on that a little bit and give us some idea of what the value is and the basis for setting it?

DR. DACRE: I'm sorry but I don't think I could. All I can say at this stage is that there is a paper describing the method with details of deriving the SPLV's in the course of preparation and will shortly be published. I assume that you've seen the abstract in the society proceedings.

DR. MURPHY: I think probably the emotional aspects of standards aren't confined to the military. It's anyplace that it is discussed. And I would like to just raise another thought with regard to comments of Dr. Back. Rather than considering various

guidelines for setting standards on the basis of classes of chemicals, what about using the basis of classes of action? I have a feeling from group discussions that I've been involved in that this may be the direction that this will take in that if you can class a chemical as a carcinogen, you deal with it in a different manner. I'm not sure anyone's decided how but it's a different matter if it turns out to produce reversible effects or irreversible effects but not progressive as cancer development is. That might be an approach that can be used for the consideration or assignment of safety factors.

DR. FRIESS: That was what I was trying to provoke in this discussion, that safety factors to classes of action and intensity of action should be related ultimately down to the degree of irreversibility. As I probe the federal structure of which I am a part, I find that irreversibility is the thing that regulators are most afraid of. The factors of 1000 appeared to be applied to actions which are irreversible at the level of a particular target organ which can lead to a degradation of the whole organism. On that basis, do you think, Dr. Murphy, that it is conceivable that classes of safety factors might be related to kinds, intensities, and degrees of reversibility of action?

DR. MURPHY: Well, I obviously think that's the direction in which we are moving or I wouldn't have made the comment. My comments were partly based on, as you probably know, some considerations related to a National Academy of Sciences panel on drinking water and a subpanel which has been involved in this question of extrapolation of animal data to standards. We have considered standards in terms of types of actions. I think what this really says, if you are going to go in that direction, you really have to have a lot more information about how various chemicals cause their effect. You just aren't going to get by with simple data such as shortening of lifetime or mortality. It's going to require fairly detailed information to change from the logic that we now use for a hundred-fold safety factor. That's not totally illogical or without any realistic thought, because as I understand it, it was based on the fact that there was seldom more than a ten-fold variation amongst several species tested. It was anticipated that there wouldn't be more than a ten-fold variation among men and, therefore, ten times ten made 100 and that's the rationale for the 100-fold safety factor.

DR. FRIESS: Dr. Dacre, in this process of applying safety factors to an evaluated syndrome in a sensitive species, we come back to man and think of classes of sensitivity. I would like to ask whether the Army has established a position with respect to whether the safety factor should be based on the most sensitized element of the population, on the average kind of population, or on the 100% healthy young military man. How do you define the philosophy with which you apply safety factors to populations which have spreads of sensitivity?

DR. DACRE: I don't know if that has been considered in detail. As Dr. Murphy pointed out, there is a ten-fold spread to allow for that suspected sensitivity. I think the problem would be a lot more difficult in the case of a possible carcinogenic compound. To get a federal agency to decide whether a compound is carcinogenic is probably going to be a very difficult task. I don't know what's going to be done about exposure standards for a lot of these carcinogenic compounds. I guess that the Department of Labor published lists of carcinogenic compounds will eventually be banned completely from manufacture. As you well know, NCI has a very extensive bioassay program underway. Quite a few of the Army compounds unique to Army use are, in fact, being evaluated in this bioassay program. If they turn out to be carcinogenic, I don't know what the Army will do.

DR. HENDERSON: I might as well stir the pot a little bit more and ask the question if a compound is highly fetotoxic, will you set a standard for industrial or military industrial operations that will protect the person who may become pregnant or will you only allow males to work in that situation, in view of our present EEOC regulations?

COL. STEINBERG (Department of the Army, Office of the Surgeon General): You do stir well, don't you? We have had test cases specifically where we attempted to not have women work on the production line. I think that nitroglycerine manufacture was a good example of this action. We were told by EEO that we couldn't do that. I think there are other examples of this problem. A good example several years ago involved freon where we were one of the major users of freon in the United States and we had some women who were succumbing to the CNS effects of the freon. We were keeping the solvent concentrations below the TLV but the TLV proved to be inadequate at the time. We wanted to move women off the production line; this was before the days of EEO, but we were not able to do so there. I should imagine that we

will have to be cognizant of the fact that we do have women of childbearing age on our munition production lines. And if the material to which the women are exposed is one that is such that one would tend to anticipate significant problems, we would have to build that into the standard. Let's face it, the military has to follow OSHA regulations just as the civilian sector does.

DR. MURPHY: Captain Kurtz, with respect to your report on the behavioral effects, I believe Dr. Steinberg mentioned that there appeared to be a problem when moban and malathion were used together or in close proximity. Did you combine the exposures in any of your experimental work?

CAPT. KURTZ (U.S. Army Environmental Hygiene Agency): No, I believe that the work Colonel Steinberg was mentioning was a comparison of the effects of moban and malathion. I don't think this involved any combination of the two. My work with animals has not involved the combination of moban and malathion.

DR. MURPHY: I guess I misunderstood Colonel Steinberg's comments. In regard to your presentation, you mentioned that lice had become resistant to DDT and lindane. Have they also become resistant to malathion now?

COL. STEINBERG: When we were working on these insecticides, there were two reports in the literature on the resistance of lice. One was in Virunde and the other was also in Africa, but I don't recall where. In addition, we had an entomologist who ran a resistance study with plate tests, and he was able to demonstrate that the Korean wild lice were beginning to show some resistance to malathion but not enough to get terribly excited about. By the way, the two insecticides are not used in combination. They were used singly when we dusted the people. You would have to experience seeing a Korean prison to realize how well separated the groups were. They have absolutely no contact one with the other. So there was no possibility of mixture.

DR. SMITH (National Institute for Occupational Safety and Health): I'd like to touch on about three items if I may since we at NIOSH are in the standards recommending business. First, the question was raised about what kind of a protection factor does one use. We debated this and considered it in many cases.

It's almost impossible to come down with a single concept, if you will, of setting a protection factor because there are so many things involved with each individual compound that you are considering. Another point that one has to keep in mind: a standard is only good if you can measure it. It's useless to recommend a standard at a level so low that you do not have an effective analytical method capable of measuring it. In addition to that, you've got to look at the practical side of life sometimes. We're in the area of recommending standards for occupational exposure. In most cases, people don't have mass spectrographs sitting around where they can take samples and measure chemical concentrations in micro and submicro quantities. Frequently we find that we are dealing with a no-effect level or a marginal effect level, if you will, at a point where you have limited capability of even measuring the concentration at that point. So what does one recommend? We have considered a number of approaches toward recommending standards both for individual compounds and for class effects such as considering aniline and aniline-like compounds. There's also another area which we have to think about. Certain occupational settings seem to be appropriate for setting standards specific for a given occupational group. Some occupations have problems peculiar to them where a standard seems to fit very nicely while others do not. I had these three items in mind and I hope I've given you a little bit of food for thought.

DR. BACK: Dr. McCreesh, I may have missed something in your talk. What about the CNS effects from the carborane compounds that you studied. Did you see any central action in the dog, for instance?

DR. MC CREESEH (U.S. Army Environmental Hygiene Agency): We conducted our studies on anesthetized dogs. However, during our six-week animal exposures to aerosol concentrations up to 200 mg/m³, we added rats to our studies. At the end of a week of exposure, we tested these rats with reward response situations and found no decrement in behavioral responses. That was the extent of the behavior testing we conducted.

DR. BACK: You don't see any chronic depression?

DR. MC CREESEH: We saw no change from control animals that were similarly exposed to clean air in the chambers.

DR. BACK: From the chemical structure, I would have expected some of these compounds to be CNS depressants with repeated exposure.

DR. MC CREECH: At lethal levels, we saw lethargy in some of these animals. This may have resulted from the pulmonary crises that developed.

DR. FRIESS: Dr. Dacre, the important new concept of soil pollutant limit values stretches way beyond the military. It becomes an interesting concept of preservation of long-term public health to prevent deposition at the factory level and beyond in soil and being absorbed by innocent population through leaching into water supplies. Did you say that in your publication which is just emerging that you have put forward a philosophy which relates to how you generate standards that would apply in the civilian community at the fence line of a factory, for example?

DR. DACRE: In deriving these values, I think we've considered all of the possible pathways that a compound can travel to get into the soil. The final pathway will be the one selected for the calculation. Not all of the compounds we've considered, you might have realized, are unique to military usage. We considered these compounds because they were identified as being important soil contaminants on a particular army arsenal. We also considered mercury chloride salts. I have a slide here of the poster presentation that was made at the SOT meeting last March. It's not a very good slide but perhaps it could be shown. It might show up some of the standards that have been derived and our method of pathways used to calculate these values.

DR. FRIESS: The question I would raise is whether you have to have a different limit value for each kind of soil drainage. Would you have limits in the arid desert regions which are a lot different from those in a valley drainage system, for example?

DR. DACRE: This is taken into consideration but we are more concerned about the actual pathways of the compounds, whether they pass through birds, fish, man and other pathways.

DR. FRIESS: That bears on the nature, then, of what enters the soil.

DR. DACRE: Yes. As I say, this slide was from a poster presentation made at the SOT meeting. If you can read it, I think it might answer some of the questions that Dr. Friess and Dr. Henderson raised.

DR. HODGE (University of California, San Francisco): Can I ask Dr. Dacre to read some of the important lines to us?

DR. DACRE: In this corner of the slide, we have the abstract which was published in the journal. We also have our procedures for establishing SPLV's. It's essentially a compilation of the physical, chemical and toxicological properties and ecological effects. The useful data was then extracted and the ADI (acceptable daily intake) is calculated. We have various transfer factors, called K factors, that we use depending on the various pathways that the particular contaminant might travel. We've tried to set out here on the slide some of the pathways that a particular compound might travel from soil to eventually get into man. The Army was particularly interested in development of this concept for one very good reason. I think the reason is still valid. Some Army arsenals are very large pieces of real estate and considerable pressure is being brought to bear upon the government to sell or transfer some of its land for various purposes such as housing developments, to extend Air Force runways and things like that. In all probability the soil on that property might have been contaminated with various compounds and various toxic agents. The Army wants to be particularly careful that the soil on the land released does not contain any toxic components. That is why we have started to define the actual SPLV which would be the safety level. I think I can answer Dr. Henderson's question. The SPLV for mercury and mercury salt has been set at 0.056 ppm in the soil. For arsenic compounds occurring in the soil, an SPLV of 1.2 ppm has been derived. For chloride salts, which manifest their toxicity mainly through adsorption by plants, a slightly higher SPLV of 3.1 ppm has been set. Now you are all well aware that the organochlorine pesticides are persistent compounds both in humans and in soil. These were calculated and set because Rocky Mountain Arsenal has a very large shell manufacturing facility where almost all of the organic chlorines (dieldrin, aldrin, and endrin) that have been produced in the United States have, in fact, been manufactured. There have been considerable pollution problems on Rocky Mountain Arsenal because of these organic chlorine pesticides. We have put SPLV values on those compounds as well. We tried to demonstrate the calculations governing these SPLV's.

They are also shown on this slide but I can't read them. This information will be published very soon. We are also in the process of setting SPLV's for additional compounds for which we have already published problem definition studies. Again, they include unique Army compounds as well as many other industrial pollutants on Army arsenals.

DR. FRIESS: There's one piece of information that I lack and that is an understanding of how the number which is set for the limit in the soil relates to the exposure concentration you are going to permit the human contacting the soil to receive. Is it such that what is set as a limit in the soil is related in some way to the TLV that a human could receive from being in contact with soil or some other limiting factor? How is the TLV related to the number that you set for the soil?

DR. DACRE: This figure is strictly related to the amount of chemical that a human might be exposed to in the soil, by direct contact, by all possible routes of getting the chemical from the soil to the human.

DR. FRIESS: What is the summation of what you say that humans should receive?

DR. DACRE: That is the SPLV.

DR. FRIESS: But the SPLV is the number which applies to the amount in the soil. What is the relationship between that number and what the human might be permitted to get from the soil by all routes? That's what I can't understand.

DR. DACRE: What you are thinking about is the ADI or acceptable daily intake. We are not sure how the SPLV relates to possible ADI's. At the present time, we don't even know if they relate to TLV's. They shouldn't relate to TLV's because, as you know, the TLV is derived under very special circumstances of exposure.

DR. S. MURPHY: Of course, you would have airborne particles, etc. It would seem that a really essential part of the data base would be the concentration of the materials in plants and the uptake of these materials from the soil into edible plants both for livestock and man. How strong is that data base?

DR. DACRE: It's well known. We tried to indicate that here on the slide. There is a column reading "Bioaccumulation." Can you read it? Mercury and mercury salts are mainly given to humans via food chains. I think it's pretty well accepted that the residuals of organochlorine pesticides travel via food chains into the humans and reside in fat depots. Some of the compounds that we've considered such as the chloride salts and some of the degradation products of GB which are shown here don't bioaccumulate. This is all taken into consideration in the calculation.

DR. HODGE: One of the pressing problems for the future, if not for the present, is the matter of the penetration of soil contaminants into underlying aquifers which certainly are going to be one of our important sources of water. Are those processes also taken into account for calculation of an SPLV?

DR. DACRE: Two of the compounds shown on that slide were DIMP and DCPD. They have both of these compounds in the ground water at Rocky Mountain Arsenal and in the aquifer and they are leaching off the military base. These are the two compounds which are in litigation brought against the Army. We're very concerned about the fact that these compounds do get into the atmosphere.

DR. FRIESS: I'd like to clarify one more time that the human exposed to soil in which there is a contaminant may then receive by all possible routes from the soil, and the aquifers, and skin contact, and the food chain, no more than the summation of intake which is less than ADI. Would that be the general rule? The number that you apply to soil is such that, from that number all possible routes of going from soil to man will result in less than an acceptable daily intake?

DR. DACRE: I would say it would be considerably less than an acceptable ADI.

CAPT. SHINGLER (Space and Missile Systems Organization): Are we only interested in taking man into consideration in the setting of SPLV's or are we also talking about protecting the plants in the area, too? For example, if we sufficiently alter the composition of the soil, we will be changing the ability of that soil to grow certain types of crops.

DR. DACRE: No, SPLV's are only concerned with protecting man. We have studies underway on the effect of a lot of munition waste pollutants on vegetation. I don't know how we will treat that information at this stage.

CAPT. SHINGLER: SPLV's would be proposed only for the protection of individuals and not as an environmental criteria. We are getting involved in environmental contamination problems quite a bit now within some of our Air Force programs where there is a possibility or an allegation that we could be changing the composition of the soil in the environment itself. We must predict how our activities would impact on the soil micro-organisms and higher food chains and the crops that could be grown in that area. That's something that we are very much concerned with right now. I think that environmental quality should be considered along with your criteria and limits that you've set for the protection of individuals.

COL. STEINBERG: One thing to keep in mind, the SPLV's are not limits for exposures that might potentially occur. The material is already in the soil. The problem is how far should we reduce the contamination level so that it is safe. In terms of changing the plant life, it's already happened. It's a clean up situation. At what point can we say that this land is clean enough now that we can hand this back to the civilian community to use for whatever purpose it should like, including a park if that's the desire of the civilian community.

DR. YOUNG (Armed Forces Radiobiology Research Institute): I just have one question along this line. Is Dr. Dacre's area of consideration limited to just chemicals or is it radioactive materials, or are they specifically excluded? Are there any plans for looking at radioactive materials if they are part of your area of responsibility?

DR. DACRE: Radioactive materials have not been considered by us. I'm not sure whether we will consider those.

CAPT. DAVIS (6570 Aerospace Medical Research Laboratory): I have a question for Dr. Kurtz. You showed a positive correlation between inhibition of brain cholinesterase and a decrement in the conditioned avoidance response. Do you know how inhibition of the tissue cholinesterase at a neural muscular junction would correlate with changes in motor activity?

CAPT. KURTZ (U.S. Army Environmental Hygiene Agency): I don't have specific information on that but I think it's a highly likely possibility that they are correlated. I cannot substantiate this by hypothesis, but I think it would not be surprising if we were to find that in fact the motor activity depression which we have observed with moban which was also accompanied by peripheral blood plasma and erythrocyte cholinesterase inhibition. These two, in fact, might be related and it may, in fact, be due to inhibition of activity of the neuro muscular junction.

DR. BACK: I'd also like to ask a question in that regard. I was surprised when you made a statement that no one could tell that these animals had been exposed. When you got 50% reduction in cholinesterase activity, didn't you see muscular fasciculations?

CAPT. KURTZ: Which data are you referring to, sir?

DR. BACK: The data on moban.

CAPT. KURTZ: Yes, we found with 5 mg/kg there were observable signs of toxicity including muscle fasciculation. When I made the statement about independent observers not being able to tell the difference between the treated animals and the normal, untreated animals, I was referring specifically to the 2 and 3 mg/kg doses of moban. If you remember that data, we didn't find as much as 50% inhibition of cholinesterase activity.

DR. BACK: When I was looking at your photo of motor activity and CNS activity, I thought you got about 50% cholinesterase inhibition when your motor activity and CNS activation were disrupted. It looked to me like it was about 50% inhibition. I would have expected that you would have seen lacrimation and that you should have seen some salivation at these levels. If you didn't, then I wonder why you didn't.

CAPT. KURTZ: With moban dose levels of 5 mg/kg, we did find that degree of cholinesterase inhibition that you're talking about, 40 or 50% reduction from control values. It may be surprising, however, we did have independent observers come in and there were animals that had been treated with these doses and some that had not. They were not able to tell the difference between the animals, which ones had been treated and which ones had not. It was a blind sort of test.

COL. STEINBERG: We've seen this response before with other insecticides. One can give a high dose with significant cholinesterase depression as a function of time and not see these physical signs.

AMRL-TR-76-125

SESSION IV

EVALUATION OF ORGAN TOXICITY IN VIVO: LIVER

Chairman

Ronald C. Shank, Ph.D.
Associate Professor of Toxicology
Departments of Community and
Environmental Medicine & Medical
Pharmacology and Therapeutics
University of California, Irvine
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PAPER NO. 16

STRUCTURAL AND FUNCTIONAL ALTERATIONS OF
LIVER CELLS IN DISEASE

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San Francisco, California

Text of this presentation is not available for publication.

SERUM ALPHA FETOPROTEIN AS AN INDICATOR
OF LIVER CELL INJURY*

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INTRODUCTION

Alpha fetoprotein (AFP) is a serum protein, the production of which is associated with normal neonatal and fetal development, restitutive proliferation following liver cell necrosis, exposure to chemical hepatocarcinogens, and growth of hepatocellular tumors. Although earlier studies established that the fetus contained some serum proteins not readily identifiable in adult serum (Pederson, 1944; Bergstrand and Czar, 1956), it was the observations of the outstanding Soviet investigator, Garri I. Abelev in 1963, that clearly identified the association of a newly identified protein with normal pregnancy and tumor growth. He described a serum protein present in pregnant animals and in animals bearing transplantable hepatomas which he designated alpha fetoprotein. Subsequently, he and his collaborators, working under difficult conditions, extended their observations on AFP production in normal and abnormal situations (Abelev, 1968, 1971, 1974). Tartarinov (1964) detected AFP in a human with a primary hepatocellular carcinoma and Stanislowski-Birencweig et al. (1967) found that animals with primary carcinogen induced hepatomas also had detectable AFP in their sera. With the development of more sensitive and accurate assays (Ruoslahti, 1971; Sell, 1973a; Sell and Gord, 1973), AFP production has now been associated with a variety of liver cell injuries, and the extent of production appears to be affected by a number of regulatory factors (Sell et al., 1976). This presentation reviews our observations on AFP production in the rat with particular emphasis on evaluation of liver cell toxicity.

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AFP is a serum protein with a molecular weight of 70,000 and a carbohydrate content of approximately 4% (Watabe, 1974; Ruoslahti et al., 1974). Amino acid sequence analysis suggests homologies with serum albumin (Ruoslahti and Terry, 1976). Different forms of AFP may be detected by polyacrylamide gel electrophoresis which are due to differences in carbohydrate content (Alpert et al., 1972; Smith and Kelleher, 1973; Watabe, 1974; Cittanova et al., 1974; Watanabe et al., 1975; Lai et al., 1976). However, the CHO content does not affect AFP immunochemically.

A specific biologic function for AFP has not been demonstrated. Three major hypotheses have been offered: 1) since murine AFP binds estrogen (Uriel et al., 1972, 1973; Savu et al., 1972), AFP may protect the fetus from the effects of maternal estrogen; 2) AFP has been reported to inhibit some immune reactions *in vitro* (Parmely and Hsu, 1973; Parmely and Thompson, 1974; Murgita and Tomasi, 1975a, b); therefore, AFP may serve as a means of protecting the fetus from maternal immune attack; 3) AFP serum concentrations fall as albumin concentrations rise late in fetal development (Gitlin and Boseman, 1967; Abelev, 1971), so it is postulated that AFP serves as fetal albumin. The first two hypotheses are not supported by other data as the AFP of nonmurine species does not bind estrogen (Nunez et al., 1974), and AFP is not found to be uniformly immunosuppressive *in vitro* and has no effect on immune reactions *in vivo*. The third hypothesis may be valid but is unsatisfying because it does not explain why there is a special protein produced by the fetus to perform the function of albumin.

The serum concentrations of AFP in the rat under various normal and experimental conditions are presented in Figure 1. It is apparent from a brief perusal of this figure that the stimulation of AFP synthesis occurs under very interesting circumstances. The following is a more detailed description of these results.

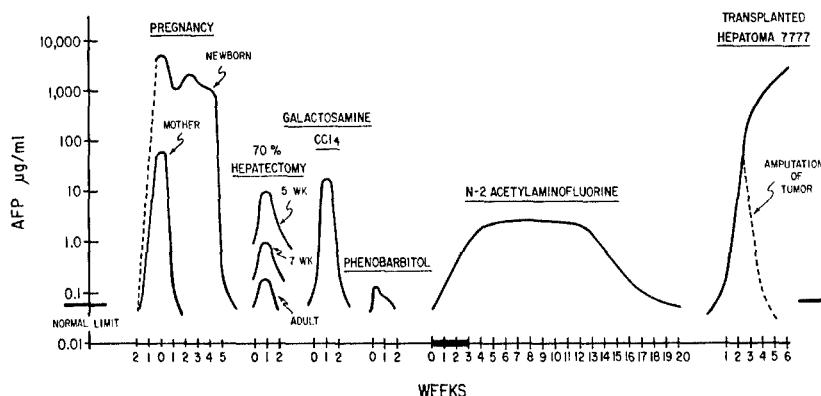


Figure 1. Serum alpha fetoprotein concentrations in rats.

AFP IN NORMAL DEVELOPMENT

In the adult rat there is a low normal serum concentration of 0.01-0.06 µg/ml (Sell and Gord, 1973), but the newborn rat has a concentration as high as 5,000 µg/ml (Sell et al., 1974). AFP first appears in the serum of the pregnant rat at 9 days prior to delivery (Sell, 1973) when fetal organogenesis is taking place. Maternal AFP serum concentrations rise until delivery, then they fall to normal levels within 10 days. AFP is synthesized by the fetal liver and by yolk sac cells (Gitlin and Boesman, 1967; Sell and Skelly, 1976). Maternal serum AFP is acquired by transfer from the fetal unit (Sell and Alexander, 1974) so that following delivery there is an abrupt fall in the maternal serum AFP concentration with a half-life of 24 hours. In the newborn rat, the temporary fall in serum AFP concentration is presumably due to the loss of the yolk sac as a site of synthesis.

In the human the serum concentration of AFP of the fetus peaks at 3,000 µg/ml at 15 weeks of gestation and then declines to about 30 µg/ml at birth (Gitlin, 1975). Maternal serum AFP concentration reflects this pattern, reaching a peak of up to 0.5 µg/ml at 28-32 weeks of pregnancy (Seppala, 1975). Human amniotic fluid AFP concentration peaks at about 30 µg/ml at 14-16 weeks of pregnancy and then falls steadily to 0.1 µg/ml at birth. Abnormally high amniotic fluid AFP concentrations may occur if there is leakage of fetal fluids. This is most frequently associated with neural tube defects, intrauterine fetal death, or congenital

nephrosis, and determination of amniotic fluid AFP concentration may be useful in diagnosis of these conditions (Seppala, 1975). Elevated concentrations strongly suggest fetal death or abnormality, but normal concentrations do not rule these out.

The newborn rat continues to have high (> 1,000 µg/ml) serum concentrations of AFP until approximately 4 weeks of age, when there is an abrupt drop to adult levels (Sell et al., 1974). The production of AFP by the neonatal rat is associated with continued proliferation of the liver; when liver cell proliferation stops, AFP production drops. A close association between fetal rat liver cell proliferation and AFP production has also been demonstrated *in vitro* (Leffert and Sell, 1974; Sell et al., 1975). AFP appears to be synthesized early in the cell cycle (prior to DNA synthesis) and released late in the cycle (prior to mitosis). Quiescent liver fetal liver cells *in vitro* do not release measurable amounts of AFP, whereas albumin production by proliferating or quiescent cultures is essentially the same (Sell et al., 1975). In the newborn human, continued elevation of AFP is associated with hereditary tyrosinemia, a liver disease which features abnormalities in methionine metabolism, cirrhosis and a high incidence of hepatoma (Belanger et al., 1973; Belanger, 1973). In addition, serum AFP concentrations may be elevated in association with ataxia telangiectasia (Waldman and McIntire, 1972).

AFP AND LIVER REGENERATION

Elevated serum concentrations of AFP are found following 70% partial hepatectomy of the adult rat (Abelev, 1971; Sell et al., 1974). Seventy percent hepatectomy results in waves of liver cell proliferation beginning approximately 24 hours after operation (Grisham, 1962; Bucher and Malt, 1971). Serum AFP becomes elevated about 48 hours after operation, reaches a peak at 3-4 days, and returns to normal within 10 days. The degree of elevation is related to age as young rats (5-7 weeks) with higher normal serum AFP concentrations respond with higher posthepatectomy concentrations than adult rats (Figure 1).

Similar events occur following the administration of agents which produce liver cell necrosis such as CCl₄, D-galactosamine, or thioacetamide. In a comparison of various hepatotoxins on AFP production by Watanabe et al. (1976), CCl₄ administration resulted in higher serum concentrations of AFP than other hepatonecrotic agents. However, the degree of liver cell damage, as measured by

SGOT activities, was also greater after CCl_4 than after the other agents used. The elevation of serum AFP found following galactosamine administration is directly related to the extent of liver cell damage (Sell et al., 1976). There is a very close statistical correlation between the highest SGOT and SGPT serum activity found 1 to 2 days after induction of necrosis and the subsequent elevation of AFP at 2 to 6 days. The elevations of AFP observed following administration of necrotic agents are associated in time with restitutive proliferation of the damaged liver and usually return to normal within 10 days or 2 weeks following the insult. When damage is extensive and fatal, serum AFP concentrations may be increasing when the animal dies. The fact that administration of hepatotoxic agents produces higher serum AFP concentrations than those observed after partial hepatectomy suggests that there may be more extensive proliferation after chemical injury or that additional mechanisms appear to stimulate AFP synthesis after chemical injury.

AFP AND CHEMICAL HEPATOCARCINOGENESIS

Although there may be liver cell necrosis following administration of hepatocarcinogens, the production of AFP induced by carcinogens is not necessarily related to restitutive proliferation. The exposure of susceptible rats to chemical hepatocarcinogens results in a sequence of changes finally culminating in development of hepatomas (Miller, 1970; Farber, 1973; Becker, 1975; Heidelberger, 1975). High doses of the carcinogen may be toxic, leading to some cellular necrosis. However, the kinetics of serum AFP elevations is quite different from that observed with noncarcinogenic toxins. For instance, the administration of 3-M-DAB produces liver cell necrosis followed by restitutive proliferation and later by carcinogenic changes (Becker et al., 1975). The early proliferation produces a low, temporary elevation of AFP. This prolonged elevation occurs at a time when little or no detectable liver cell proliferation is occurring.

In addition, administration of carcinogens at doses insufficient to produce liver cell damage induced a prompt and prolonged elevation of serum AFP (Becker and Sell, 1974). In fact, the production of AFP may be the earliest systemic event now detectable as an indication of the reaction of hepatocytes to the effects of a chemical carcinogen. Although earlier studies indicated that AFP serum concentrations became elevated after carcinogen exposure, prior to hepatoma development, the

methods used for AFP measurement did not detect the earliest changes (Watabe, 1971; Kores et al., 1972). Following administration of 3-M-DAB, Dempo et al. (1975) found AFP in periportal hepatocytes termed transitional cells by immunofluorescence. However, we have demonstrated that AFP elevations occur in situations where no morphologic alteration in the liver can be seen. Significant elevations of AFP may be seen as early as 45 hours after the administration of ethionine in the absence of evidence of liver cell necrosis or other significant morphologic changes (Smuckler et al., 1976). More extensive studies have been carried out on the effects of feeding 2-FAA to rats of different strains and sex. A pulse dietary feeding schedule of 2-FAA was developed by Teebor and Becker (1973), to avoid some of the toxic effects of continuous carcinogen administration. The diet containing 2-FAA is fed for 3 weeks followed by 1 week on normal diet. With a 3 week on 1 week off cycle, neoplastic nodules appear after 4 cycles (app. 16 weeks) and hepatocellular carcinomas are first seen after 5 cycles. Using such protocols it was found that elevation of serum AFP occurred within one week of feeding a carcinogenic dose of 2-FAA (Becker and Sell, 1974). After cessation of the diet (one 3 week pulse), the serum concentrations of AFP remained elevated for an additional 9 weeks and did not return to normal until 20 weeks after the diet was first fed (see Figure 1). In addition, serum AFP elevations were produced by subcarcinogenic doses of 2-FAA in susceptible male mice and in female mice which are resistant to the carcinogenic effects of 2-FAA (Becker and Sell, 1974). These results again demonstrate that elevations of serum AFP may occur in the absence of detectable histologic changes in the liver.

There is no evidence that animals which are genetically susceptible to development of hepatocellular tumors have abnormal serum AFP concentrations. The normal adult serum AFP concentrations of mice strains vary considerably (Pihko and Ruoslahti, 1973), but strains with the higher serum concentrations do not have an increased incidence of spontaneous hepatomas. In addition, the serum AFP concentrations of strain C₃H-AVY, which spontaneously develop hepatocellular carcinomas with aging, do not become elevated with aging alone; i.e., elevations do not occur until tumors actually appear (Becker and Sell, 1977). Therefore, elevated serum AFP concentrations do not indicate genetic predisposition to tumor development but may suggest exposure to a chemical hepatocarcinogen or the presence of a growing tumor.

PHENOBARBITAL

The appearance of AFP in the serum of rats injected with phenobarbital, which is not a carcinogen, suggests a third mechanism of activation of AFP production (Smuckler et al., 1976). Administration of phenobarbital results in an increased serum concentration by AFP within 6 hours. This elevation occurs prior to the mitosis induced by phenobarbital, and is transient with a return to normal within 12 hours after phenobarbital injection. However, a second elevation is seen at 36 hours which follows a wave of mitosis which occurs at 21 hours. Thus, the altered phenotypic expression induced by phenobarbital may occur not only associated with the induction of proliferation but also as the result of hypertrophy without division.

HORMONAL REGULATION

Further studies carried out in collaboration with Hyam Leffert of the Salk Institute suggest that the amount of AFP produced by proliferating fetal liver cells in vitro is affected by hormones (Leffert et al., 1977). Since the same hormones also affect hepatocyte proliferation, it is difficult to differentiate primary effects on protein synthesis from secondary effects because of alterations in the rate of cell division. For instance, when the amount of protein in culture media is measured it appears that hydrocortisone suppresses AFP production and stimulates albumin production. Insulin and glucagon synergistically stimulate AFP production much more than albumin production. However, when the data are expressed as the average amount of specific protein released throughout the growth of the cultures, it is seen that hydrocortisone slightly stimulates AFP production whereas albumin production is markedly stimulated. On the other hand, insulin and glucagon combined markedly increase the amount of AFP released per cell, but have little or no effect on albumin release. These studies show that hormones which control certain aspects of hepatocytic proliferation also control differential expression of protein production.

AFP PRODUCTION BY HEPATOCELLULAR CARCINOMAS

The serum concentration of AFP in rats bearing transplantable hepatomas is extremely variable (Sell and Morris, 1974). Some hepatomas produce elevations as high as 15,000 $\mu\text{g}/\text{ml}$, whereas others produce no elevation. The ability of a given hepatoma to

produce AFP is associated with fast growth rate and increased degree of chromosomal abnormality, but important exceptions have been found (Sell and Morris, 1974; Becker et al., 1975). For instance, Morris hepatoma 7777, which is fast growing but has only one extra chromosome, produces very high AFP elevations, and hepatoma 9098, which is fast growing but essentially diploid, does not produce abnormal serum AFP concentrations. If a tumor does produce AFP, the serum concentration of AFP may be used as an accurate index of the growth of the tumor (Figure 1). Such a model system has been used to study the effects of various therapeutic procedures on the growth of a transplantable hepatoma (Sell et al., 1975, 1976a, 1976b). It is of particular importance to note that many primary hepatocellular carcinomas induced by chemical carcinogens do not produce elevated serum AFP concentrations (Becker et al., 1973) even though every rat treated with hepatocarcinogens has sustained elevations of AFP early after carcinogen exposure (Becker and Sell, 1974; Kroes et al., 1975).

AFP IN HUMANS

Serum AFP concentrations have been used to follow tumor growth and liver injury in humans (Abelev 1968, 1971, 1974; Masseyeff, 1972; Ruoslahti et al., 1974; Sell and Wepsic, 1975). The normal serum concentration of AFP in humans is 20 ng/ml. In order to evaluate the clinical significance of serum AFP elevations, serial determinations must be carried out. Low transitory elevations may occur with liver injury (such as hepatitis or chemical injury) (Abelev, 1974; Silver et al., 1974; Endo et al., 1975). Sustained or steadily increasing serum concentrations suggest hepatocellular or yolk sac (endodermal sinuses) tumors (Waldman and McIntire, 1974). A lack of elevation occurring after acute liver injury is a poor prognostic sign as elevated AFP concentrations reflect restitutive proliferation. The effect of hepatocarcinogenic exposure on serum AFP concentrations in humans has not been determined.

SUMMARY

The production of alpha fetoprotein (AFP) in various experimental situations in the rat and mouse is reviewed and compared to production associated with human diseases. Production of AFP is associated with critical events in liver cells. These include

fetal growth, regenerative proliferation, and exposure to hepatocarcinogens or other agents which affect liver cell metabolism. In addition, the extent of AFP production may be regulated by hormones. Sequential determination of the serum concentrations of AFP in humans may provide useful information in diagnosis and prognosis of liver injury or tumor growth as well as abnormalities in fetal development.

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OPEN FORUM

DR. MURPHY (Harvard School of Public Health): Dr. Sell, I don't think you mentioned DDT, aldrin or dieldrin but I presume you have either some data or some opinions about that. I'd like to hear if you do. Also have you, in the clinical situation, looked into the relationship of women on contraceptive steroids and reports of neoplasia or neoplastic nodules?

DR. SELL (University of California, San Diego): I can't answer either of those questions. I think those are points that we need to look into. In regard to agents which are not necrotic agents, I think each of these has to be considered separately. The finding with phenobarbital is still preliminary, and it is quite fascinating to us. Whether other agents produce alpha-feto-protein elevations or not, I don't know. The situation with hormones is a very complicated one and we are looking at this in vitro because various combinations of hormones can seriously affect the growth of liver cells. And there is one report that dexamethasone, the artificial compound, actually suppresses alpha-feto-protein production but we have found in vitro that it also suppresses proliferation. When the number of cells present in the culture and the amount of alpha-feto-protein are compared, dexamethasone stimulates production per cell, but it inhibits cell proliferation so that the total amount made is less.

DR. DOST (Oregon State University): Dr. Smuckler, I appreciate your efforts in relating function and structure. Usually we have a relationship between structure and something like death or some other similar circumstance. What I am interested in is whether you have done any such studies at relatively low dose levels or chronic exposures with carbon tetrachloride? The reason I ask this is because we are beginning work with some pyrrolidizine alkaloids which are the active components of some poisonous plants that are metabolized to pyrrols, and at least the superficial effects are very similar to those of carbon tetrachloride including a phase of decreased protein synthesis. However, we found that when treating animals chronically, we were able to find a dose which (1) did not produce any lesions visible by light microscopy, and (2) caused increased protein

synthesis in a number of parameters, such as the incorporation of labelled leucine, in the ability of the animal to induce ornithine decarboxylase, and in tubulant synthesis. Do you have any speculation on what might happen with carbon tetrachloride and what relationship there might be between these circumstances?

DR. SMUCKLER (University of California, San Francisco): Repeated low level exposures to carbon tetrachloride, particularly if the level is chosen so that if you are below that necessary to produce significant cell death, is associated with an altered phenotypic expression in the liver cell. Although you may not see anything by light microscopy, there are indeed functional changes which take place. The livers become refractory to the effect of the carbon tetrachloride as they do to senecio alkaloids. I think you will find that if you compare the acute effects of carbon tetrachloride exposure to the sub-lethal effects of the senecio alkaloids, you will produce the same sort of peculiar panorama. What happens to these liver cells which have come to a new steady state and why they become more synthetic is not clear. This is essentially analogous to the peculiar changes that take place with phenobarbital. It's the reason we started. The question arose whether phenobarbital or low levels of senecio alkaloids or low levels of carbon tetrachloride that modify the liver could produce hyperplasia or hypertrophy, and it was unanswered. Our preconceived notion was that if you used a marker for proliferation such as alpha-feto-protein, you could differentiate between these two possibly more satisfactorily than you could either through autoradiographic assays or measurement of mitoses. It turns out, at least with the model system, that that is not true because we get alpha-feto-protein unassociated with changes in nuclear number or mitotic figures. Similarly in low level exposures to carbon tetrachloride, you get these same peculiar changes without anything definitive. The problem with labelling experiments, unless you have a specific component that you are seeking, if you are using a nucleic acid precursor, you have to differentiate between regeneration and repair. We haven't done that. In fact, if you attempt to do it utilizing the hydroxyurea system with metabolic poisons, you find you get quite a mess, particularly in the liver. The senecio alkaloid story, as you are well aware, is a very interesting one. I am not sure whether the black liver disease of sheep resembles what you are producing in rodents.

DR. DOST: Well, we had speculated that this is almost an override of response. However, we are so early into this study that the information we have is, in fact, all preliminary. One of the difficulties we face is that even with the long history

of this form of intoxication, nobody apparently has done any decent low level, long-term studies which is very difficult to understand because that often is the way the needed information is acquired.

DR. CROCKER (University of California, Irvine): I'm confused about female Sprague-Dawleys that were fed a carcinogen to which they could not respond with hepatoma induction but who had the same rise in alpha-feto-protein as the male Sprague-Dawleys that could respond in that way. If one were to take an unknown agent asking, is this a carcinogen or isn't it, and applied it in animals and found no indication of toxic damage to the liver but found alpha-feto-protein and thought that this might be a guide to the fact that there was a carcinogenic exposure, this could be misleading as I take it. This would have been in the female Sprague-Dawley rats.

DR. SELL: Actually, it is not an absolute lack of metabolism and the female rats are not completely resistant to the carcinogen. The way it's sometimes said is that if the male Sprague-Dawley rat is equated with 100% capacity to metabolize the carcinogen, then the female probably has about 10% capacity. There is some metabolism of the carcinogen in female rats and sometimes they will get hepatomas when fed these carcinogens. In fact, in our interpretation of this, the alpha-feto-protein elevation appears to be so sensitive to low doses of the carcinogen in the resistant strains that it definitely indicates exposure but it may not predict eventual tumor development. Now, the importance of the analogues comes in at this point because 4-FAA which is not carcinogenic does not produce elevations in alpha-feto-protein. We believe that the data indicates exposure while it doesn't necessarily predict which animals will get tumors. If everyone in this room was exposed and everyone had an elevated alpha-feto-protein concentration, one would expect only a small percentage of the population to develop this particular tumor if the same kind of situation held.

DR. CROCKER: Were you able to look at individual females, for example, to see whether some did and some did not develop tumors or were these average values for a number of animals?

DR. SELL: This was not done in this study because we did not feed enough carcinogen to the animals to cause hepatomas which we are now doing. This was, as you saw, a single dose

feeding study and in these situations, one would not expect to see any morphologic change in the liver at the time the AFP was elevated. The repeated dose studies are now underway, but the results are not complete.

DR. SHANK (University of California, Irvine): This is important because this AFP test has been used in the field epidemiologically to measure the incidence of human liver cancer.

DR. SELL: The evidence in humans is actually better than it is in rats. The initial studies were done in southeast Asia and whether or not the rat model resembles the human one, of course, is open to considerable question. The fact that carbon tetrachloride elevates the levels of alpha-feto-protein in the Sprague-Dawley rat where carbon tetrachloride is not a carcinogen answers in part your question. The difficulty is whether or not any one specific phenotypic expression of liver function can be correlated with neoplasia and the answer is, no. I'd just like to say that one determination will not tell you anything because in necrotic liver injury, it would go up and come down whereas in carcinogen exposure, it may stay up. The production of alpha-feto-protein is not necessarily associated with every hepatoma so it won't tell you necessarily that a patient has a hepatoma. In fact, it may be that early after exposure to a carcinogen, the AFP levels may be higher than after a chronic exposure because that's what happens with the rat.

DR. SLONIM (6570 Aerospace Medical Research Laboratory): Dr. Sell, what method do you use for analyzing alpha-feto-protein? How long does it take and what is its sensitivity? What are the approximate normal values in man and when do you worry when you have a high alpha-feto-protein?

DR. SELL: The method used is a standard immuno assay. We use at the present time the Forrer system which is a salting out system to bring down the antigen antibody complex. We have in the human a double antibody system. The assays are standard radioimmuno assays such as those used for digitoxin and so forth. It takes about 24 hours to complete the assay. The low assay takes a little longer because we have to use preincubation and postincubation of the sample in order to increase the sensitivity but that's not necessary for most determinations. The upper limit of normal in the human is somewhere between 40 and 50 nanograms per milliliter which is essentially the same as in the

rat. One has to worry about elevations above that but again serial determinations are necessary in order to determine the significance of that elevation. We once measured a value of over 1000 nanograms per ml which is 2 logs higher than normal and the patient was suspected of having a hepatoma. It turned out that he died a week later with hepatitis, and we found that they had assumed the alpha-feto-protein level we found was supportive for a diagnosis of hepatoma but they didn't get another value. You have to be careful, you can't just take one value from a patient and make a diagnosis. The somewhat ironic thing is that we have had a human assay available in our laboratory for over five years now. But there has been very little interest in running this on various human surveys. We were originally supposed to set this up to study patients at risk. But for 5 years, I guess NIH has been unable to identify any population at risk because they haven't followed through with asking us to run the assay on it. So we've gone merrily along with the animal studies.

DR. BACK (6570 Aerospace Medical Research Laboratory): I have a question about whether alpha-feto-protein values increase at the specific time that the ribosomes from rough ER are either coming off or going back on to the rough ER. Has this correlation been done? Some time ago, we did some work on other hepatotoxic compounds where we were exposing animals continuously for some 90 days. We followed the rough ER changes and watched the ribosomes fall off of the rough ER and the swelling of mitochondria and changes in the cristae and so forth. We also saw fatty changes on the EM and then watched this whole process reverse toward normal. We measured free fatty acid levels at the same time and in the face of continuous exposure the free fatty acids elevated and then they came back down to normal despite the fact that the mice were continuously exposed to the halogenated hydrocarbon. If we had measured alpha-feto-protein at the same time, would that also have elevated at a specific time associated with the time that ribosomes are supposed to be producing protein? Has any work been done in that area?

DR. SELL: The correlation between the restoration of synthesis of protein in the liver and the formation of alpha-feto-protein is quite good. In the chronic exposure, the capacity of the organ to activate the toxin decreases so that your healing process represents a competition between those processes which are destroyed and those which are restored. After exposure to either thioacetamide or to carbon tetrachloride,

which are necrogenic, or to ethionine, which is nonnecrogenic but inhibits protein synthesis, the appearance of alpha-feto-protein correlates very well with the decreased ability of the residual liver cell to metabolize either carbon tetrachloride or thioacetamide. In response to your question, I would predict that you would have a curve that would be similar but would have a lateral translocation on the abscissa to either the healing phase of your lipid removal or the reformation of protein or the restoration of the ribosomes. I don't know if they go back on. It would sure seem to fit that way.

DR. CROCKER: I want to come back to the matter of the heteroploid cell and the production of alpha-feto-protein. I gather that the small proportion of hepatomas that ultimately do occur and do associate with alpha-feto-protein production are the heteroploid ones and the diploid ones are the ones that fail to associate in that way. Is there any regular association between the conversion from diploid to heteroploid state of cells and the production of alpha-feto-protein in any cell system other than liver? For example, would it be a way to recognize heteroploidy? And is this one of the reasons why the other tumor types in man have varying proportions of patients with positive alpha-feto-protein in their sera? Is it merely because some of the tumors have not progressed to heteroploidy while others are still diploid? Is there any way to generalize on this rule?

DR. SELL: I don't know that you can generalize other tissues but the hepatoma situation is quite clear and that is that with the Morris hepatomas the diploid or near diploid tumors do not produce alpha-feto-protein. A classic example of that is the 9098 cell line which is an undifferentiated fast growing tumor. The association is with fetal tissue that can make alpha-feto-protein as I indicated. I would assume that in these tumors there are also chromosomal abnormalities. The question remains as to what kind of other tissue embryonic material will be produced by a tumor from one type of tissue. You can only take this back so far. In the production of hormones by lung tumors, for instance, these are tumors that are generally in the lineage of hormone producing tissues in various parts of the body. With alpha-feto-protein, the general rule seems to be that the tumors that produce this material are in the lineage of the fetal tissues that made alpha-feto-protein. Now whether that 7% AFP in the lung tumors is really produced by the tumor is not resolved by the study as I reported to you. Occasionally, there are reports of non-GI tumors associated

with AFP production and usually these are lung tumors. There is one report where the presence of immunofluorescence was shown to be in liver tissue surrounding metastases in which there had been destruction and proliferation of the adjacent liver tissue, whereas the tumor itself was negative. I think the question is still open as to whether a lung tumor can express the embryonic component of GI tissue. It seems to me that it could, theoretically, if there is enough retrogression of the tissue.

DR. SMUCKLER: Another interesting aspect of this is that the loss of competence for making alpha-feto-protein occurs during the shift from a diploid to a tetraploid state in the fetal or the neonatal liver. The surge of formation of alpha-feto-protein following phenobarbital stimulation is associated with a shift from the preparation of going from tetraploid to diploid during the preproliferative phase in the stimulated liver.

DR. WINSTEAD (National Academy of Sciences): I find this is a very interesting phenomenon. Would either one of you care to speculate on the mechanisms involved in the increased alpha-feto-protein production?

DR. SELL: That's what I hope to study for the next 4 years. Now that we have identified these situations, what is the molecular mechanism associated with it? The genetic information is there but this is a question that is essentially a basic question in all animal biology. What is the mechanism of control on gene expression? Here we can evidently turn the genes off and on. I can only speculate and say that perhaps there is a change in nonhistone proteins or in chromatin structure and so forth and so on, but all I'm doing is talking off the top of my head. Hopefully, if you ask this question 4 years from now, we can be a little more precise in our answer. We assume that the message is increased. There have been studies that showed that messenger activity as in the wheat germ system is increased in the situation when AFP is produced. So somehow the message is getting out. But how the message gets in to get the message out, I don't know.

AMRL-TR-76-125

SESSION V

ENVIRONMENTAL STUDIES

Chairman

Lt. Col. Roger C. Inman, USAF, VC
Chief, Environmental Quality Branch
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6570th Aerospace Medical Research
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Ohio

LABORATORY AND FIELD EVALUATION OF TWO
HCl DETECTION INSTRUMENTS

Robert C. Ligday, 1Lt, USAF
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and
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INTRODUCTION

Solid propellant rocket motors used in the Titan III series of space launch vehicles and planned for use in the Space Transportation System (Space Shuttle) contain aluminum fuel and ammonium perchlorate oxidizer mixed with an acrylic polymer binder. Upon ignition, this propellant formulation generates several toxic exhaust constituents including hydrogen chloride (HCl) gas, carbon monoxide and aluminum oxide particulate. The presence of these chemicals in the lower atmosphere has in turn generated considerable study into their atmospheric mixing and ultimate fate (Cohen, 1973).

Hydrogen chloride is the most toxic of the three main solid propellant effluents, and hence the exhaust constituent of primary environmental concern (National Academy of Sciences, 1971). In the early studies on environmental effects of large solid motor firings, HCl in the exhaust cloud was determined by bubbler sampling and wet chemical analysis (Cesta and McLouth, 1969; Smith, 1971 and 1972). This procedure proved only marginally successful due in part to sample averaging and, perhaps mainly, to difficulties in sample siting. Later attempts at a more comprehensive sampling network using pH-sensitive papers provided only qualitative estimates of the acid exposure profile (Hendel, 1972).

The USAF School of Aerospace Medicine (USAFSAM) became involved in HCl monitoring by extension of previous work involving coulometric analysis of chlorine in chemically generated breathing

oxygen (Reyes et al., 1973). Since the coulometer, in fact, converts dissolved chlorine to chloride ion prior to detection, it required only minor modification to adapt the instrument system to HCl detection. Following laboratory validation of the coulometric technique, the instrument was field tested at several locations in cooperative programs with other Air Force organizations, the National Aeronautics and Space Administration (NASA), and the Environmental Protection Agency. These tests demonstrated that the microcoulometer was an excellent tool for measuring total HCl dosage in static environments.

An airborne monitoring program at Vandenberg Air Force Base, however, required a fast-response instrument to determine instantaneous concentration changes in a short-exposure situation. Following an earlier NASA program (Gregory et al., 1974), the USAFSAM contracted with Geomet Corporation, Pomona, California to develop a chemiluminescent-HCl analyzer to meet this requirement. The resulting instrument (Sibbett et al., 1975) was field tested in a June 1975 airborne monitoring test at Vandenberg AFB, and has been subsequently used by the Air Force Rocket Propulsion Laboratory in assessing downwind HCl concentrations from static test of the MX prototype engine.

This report describes the operating characteristics of the microcoulometric and chemiluminescent HCl analyzers and summarizes results from the several laboratory and field evaluation studies.

DESCRIPTION OF EQUIPMENT

MICROCOULOMETER

Description

The microcoulometer we used for both ground and airborne monitoring of HCl has been described in a previous report (Reyes et al., 1975). Briefly, the basis for the technique is chloride precipitation by silver ion, which is electrolytically replenished at a cost in electric current proportional to the chloride ion (or HCl) dosage. Because Faraday's law applies and the reaction is stoichiometric, the microcoulometer is a primary standard for chloride determination in liquid solution. The threshold limit is about 3 ng (Reyes et al., 1975).

In the continuous air-monitoring application, the microcoulometer is limited in both response time and dynamic range. The combination largely precludes a determination of HCl concentration for steady-state exposures of more than 5 ppm for less than about 8 seconds. Figure 1 helps illustrate the problems. In the (typical) non-steady state field situation, the HCl concentration can be estimated for certain concentration-time profiles. Since the instrument response time (lag time) is approximately compensated by instrument overshoot, the average concentration can be estimated by assuming HCl is admitted only during titration rise. Measurement of the titration peak area gives total weight of chloride from which HCl volume can be calculated. Average concentration is then obtained by dividing HCl volume by the volume of sample admitted during the time interval from initial instrument response to peak apex. In laboratory experiments, this procedure has proved accurate to $\pm 10\%$ for short exposures of constant concentration.

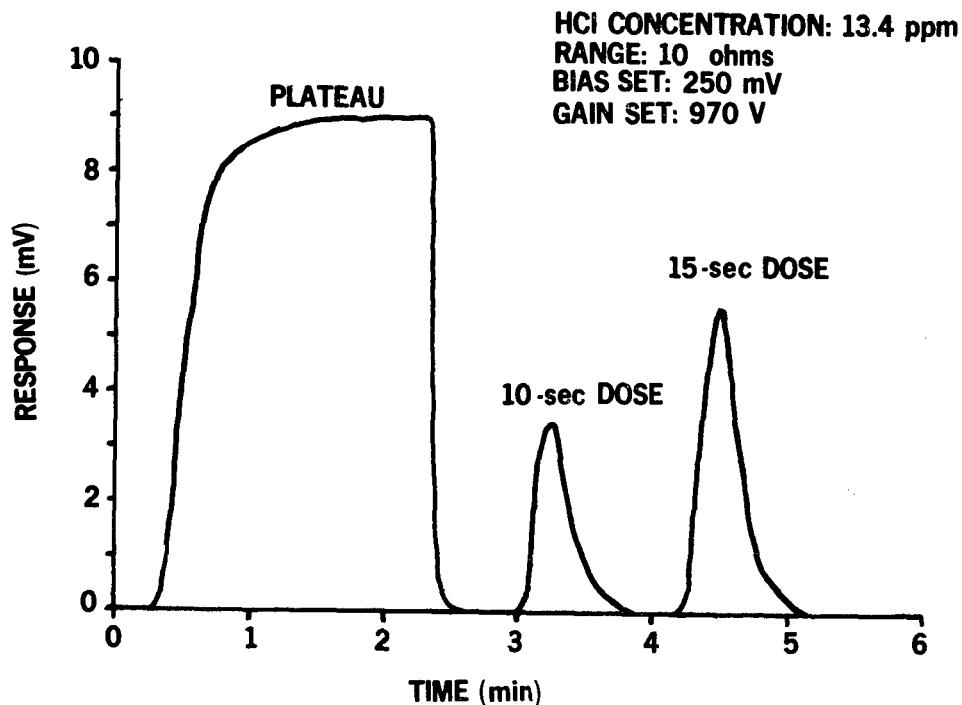


Figure 1. Typical coulometric responses to a steady HCl concentration. The combination of a high concentration and a slow response prohibits attainment of a plateau and thus concentration determination in the short exposure situation.

Calibration

The unique advantage of the microcoulometer is that it is a primary standard and thus does not require multiple point field calibration. We have routinely standardized the microcoulometer in both laboratory and field tests using a standard solution containing 20 mg/liter of sodium chloride in water. Syringe injection of 5 and 10 μ l of the standard at a given range setting generates a reproducible titration curve of known dosage.

CHEMILUMINESCENT ANALYZER

Description

Che miluminescent detection of HCl is based on exothermic oxidation of luminol (5-amino-2,3-dihydro-1,4-phthalazinedione) in alkaline solution by hypochlorous acid. The light intensity generated by this reaction is linearly proportional to HCl concentration in the incoming gas stream and is measured by a photomultiplier detector. Hypochlorous acid is formed from HCl by passing the incoming air stream through an alumina column coated with a solution of 10% sodium bromate and 10% sodium bromide. This mixture reacts with hydrogen chloride to produce hypochlorite and hypobromite which initiate luminol oxidation to generate light.

A functional block diagram of the air and fluid paths of the chemiluminescent analyzer is shown in Figure 2. Air is drawn through the bromate/bromide-coated column and reaction cell at a rate of 2 liters/minute by a diaphragm vacuum pump. Liquid reagents are fed into the reaction cell where contact is made with the air stream, resulting in a chemiluminescence intensity proportional to the HCl concentration. The air-reagent mixture is then pumped into a reservoir where the liquid waste is deposited. The effluent gas is exhausted through a flow regulation valve and flowmeter.

The two liquid reagent employed are (a) 0.3% hydrogen peroxide in 0.086% phosphoric acid, and (b) luminol (2 mg/ml) in 0.5M sodium carbonate. Both reagents are stable and are stored in separate reservoirs in the instrument case. The reagents are metered in separate lines by a constant speed-peristaltic pump and mixed in a tee immediately upstream from the reaction cell.

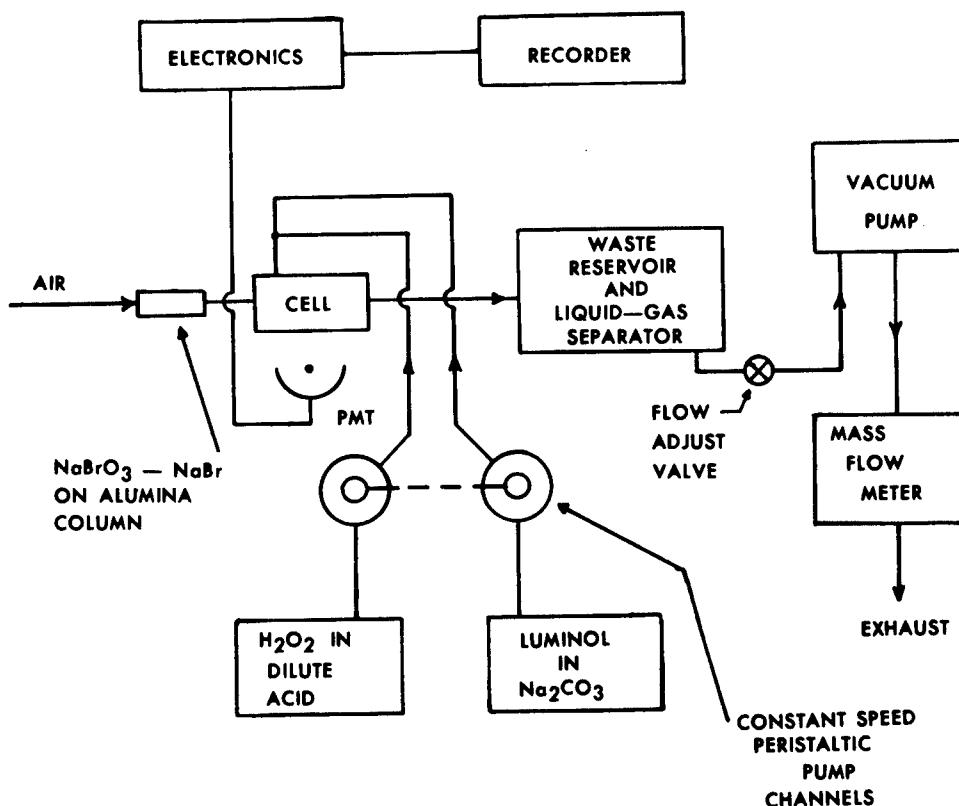


Figure 2. Functional block diagram of the air and fluid paths in the Geomet Model 402B chemiluminescent HCl monitor.

We have used three models of chemiluminescent HCl analyzers in various studies; all were developed by Geomet Corporation and embody the same luminol detection principle. The first instrument (Model 401) was designed primarily for laboratory use and contained two reaction cells, one for detection of HCl and the other for reference subtraction of interferent gases. The reference cell inlet is an uncoated alumina column, and was found to be mainly sensitive to chlorine interference in chlorine-HCl mixtures (Gregory et al., 1974).

Because of the relative bulk of the laboratory instrument, the USAFSAM contracted with Geomet Corporation in late 1974 to develop two improved chemiluminescent-HCl analyzers for different

field applications -- ground and airborne monitoring. The improved ground instrument (Model 401B) is about one-fourth the size of the Model 401 and weighs only 13 kg (30 lb), making it easily portable for field deployment. The airborne monitor (Model 402B) is a split instrument with separate control and sensor components, for ease of installation in aircraft. The 402B is designed for 28 VDC operation. The performance specifications for Models 401, 401B, and 402B instruments are shown in Table 1.

TABLE 1. PERFORMANCE SPECIFICATION FOR THE GEOMET
HYDROGEN CHLORIDE MONITORS

<u>Model</u>	<u>401</u>	<u>401B</u>	<u>402B</u>
Operating ranges, ppm	0-0.5 0-5 0-50 0-100	0-0.1 0-1 0-10 0-200	0-0.2 0-2 0-20
Threshold sensitivity, ppm	0.05	0.01	0.02
Physical dimensions, cm W/D/H	50/70/35	22/37/25	S-25/46/20 C-40/23/10
Weight, kg	22.7	13.5	S-13.6; C-4.5
Power req. VAC/watts VDC/watts	110/250 -	110/250 -	- 28/75
Unattended performance, hr	8	4	6
Linearity, ppm	0-50; $\pm 5\%$	0-50; $\pm 5\%$	0-100; $\pm 5\%$
Response time	1 second to 90% full scale		
Recovery time	1 second to 10%		
Start-up time	10 minutes		
Temperature range	0-40 C (32-104 F)		
Relative humidity range	10-95% R.H.		

Calibration

Calibration of the chemiluminescent HCl monitors has been accomplished in the laboratory by standardization with a micro-coulometer. Figure 3 illustrates the setup used in the calibration procedure. A steady concentration of HCl gas is passed simultaneously into both the coulometer and the chemiluminescent analyzer under a slight positive pressure. When the steady-state response is achieved by the coulometer, the concentration may be calculated (Reyes et al., 1975) and used to adjust the span on the chemiluminescent analyzer. We have used HCl gas mixtures

produced commercially in pressurized cylinder, and also produced in the laboratory by passing nitrogen gas over a diffusion tube in a calibration oven (Analytical Instruments Development Model 309). The diluent nitrogen may be passed through a water bubbler to humidify the gas prior to addition of HCl. A low relative humidity inhibits the chemiluminescent reaction by drying the bromate-bromide coating in the column and thus slowing the hypochlorite/hypobromite-producing reaction. In field tests, the chemiluminescent analyzer has been calibrated in the laboratory before and after each field measurement, with acceptable reproducibility (Ligday and Giannetta, 1975).

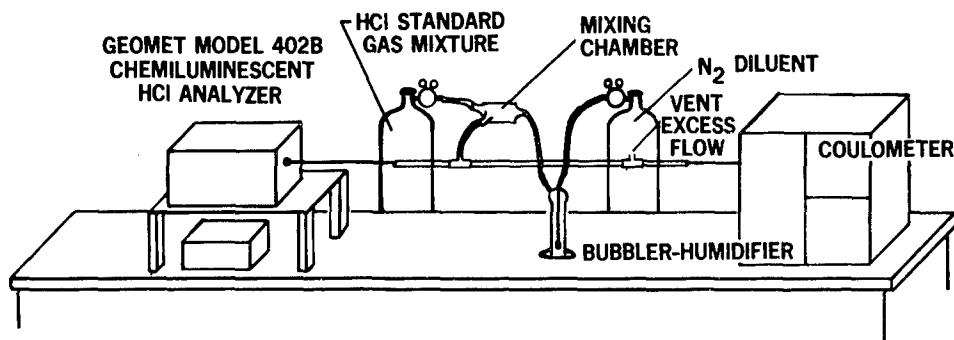


Figure 3. Geomet (Model 402B) calibration setup. Calibration of the chemiluminescent HCl monitors has been accomplished in the laboratory by standardization with a microcoulometer.

Because of the recurring need for quick standardization of the chemiluminescent analyzer, we have recently developed a syringe pump calibration technique adaptable for field use. The system consists of a syringe pump (Sage Model 341), 20 cc polypropylene syringe (Stylex) and polypropylene needle (0.5 mm ID). The HCl source is the vapor over a small quantity (1 ml) of constant boiling hydrochloric acid (20.24 percent) contained in the syringe. Slow infusion of the vapor into diluent air produces a reproducible concentration of HCl as a function of infusion rate, air flow rate and temperature. When temperature and air flow rate are constant, the HCl concentration is a function only of syringe pump infusion rate (Figure 4). Conversely, when syringe pump infusion rate and airflow rate are constant, the HCl concentration is a function only of temperature (Figure 5). With proper attention to temperature

control, calibration reproducibility of the chemiluminescent analyzer is plus or minus 10 percent of the HCl concentration determined experimentally.

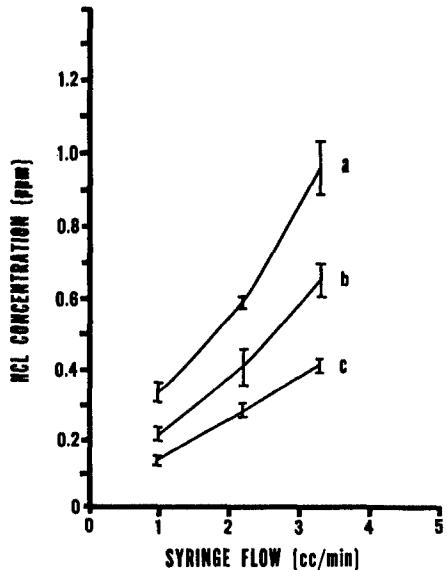


Figure 4. Syringe pump calibration technique, HCl concentration vs pump infusion rate. When temperature and air flow rate are constant, the HCl concentration is a function only of syringe pump infusion rate. Temperatures: a) 34.5 C; b) 25.5 C; c) 12.0 C.

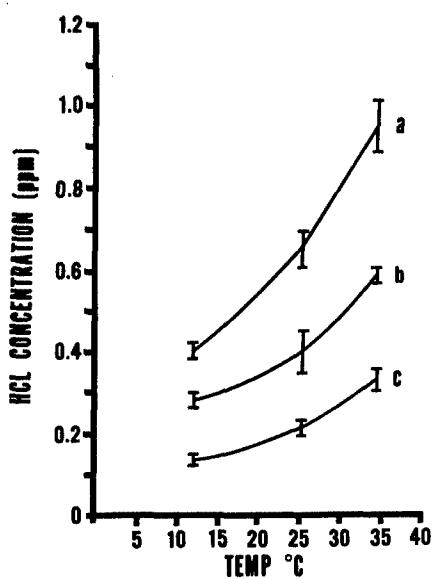


Figure 5. Syringe pump calibration technique, HCl concentration vs temperature. When syringe pump infusion rate and air flow rate are constant, the HCl concentration is a function only of temperature. Syringe flow: a) 3.3 cc/min; b) 2.2 cc/min; c) 1.0 cc/min.

FIELD EVALUATION

CAPE CANAVERAL AIR FORCE STATION

In mid-1972 the National Aeronautics and Space Administration initiated a comprehensive program of monitoring space launch vehicle effluents for the Space Shuttle program (NASA, 1972). At the request of the Langley Research Center, the USAFSAM participated in the tropospheric (ground) monitoring portion of this project. The primary objective of the program was to obtain field data for validation of the multilayer dispersion model employed by NASA to predict the behavior of launch vehicle ground exhaust. Measurement systems employed by NASA included detectors for hydrogen chloride, carbon monoxide, carbon dioxide, and aluminum oxide particulate.

Downwind effluent HCl was detected for the first time in the NASA/AF monitoring program, following the launch of a Titan IIIC vehicle from Cape Canaveral Air Force Station on 30 May 1974 (Reyes et al., 1975). The USAFSAM monitoring equipment included a microcoulometer and a chemiluminescent analyzer (Geomet Model 401), both deployed on an ocean tugboat at site P-2 (Figure 6). Figure 7 shows a reproduction of the response obtained with the microcoulometer. The average HCl concentration of 1.8 ppm (2.9 mg/m^3) was obtained at T+22.8 minutes at the position indicated on Figure 6. The detection time was confirmed by the chemiluminescent detector. The tugboat (P-2) arrived at its prelaunch holding position approximately 5 km from LC 40 at about T-2 hours. This position, immediately north of the boat-hit-probability-contour, was maintained until launch (T=0). At T+4 minutes, P-2 headed south toward its pre-assigned sampling location. Concurrent with launch, however, a southerly wind shift was detected and the vessel was vectored in a more southeasterly direction for cloud interception. At about T+19 minutes, the tugboat made contact with the outer edge of the moving exhaust cloud, approximately 5.2 km from LC-40 on an 81° azimuth. During the estimated cloud passage time of 1.9 minutes, the total HCl dosage was 454 ng (as chloride) which calculated to an average concentration of 1.8 ppm HCl. This concentration was well below the time-weighted average short-term public limit of 4 ppm for 10 minutes (National Academy of Sciences, 1971). The average HCl concentration was estimated by assuming the total chloride dose was admitted during the 1.9-minute passage time.

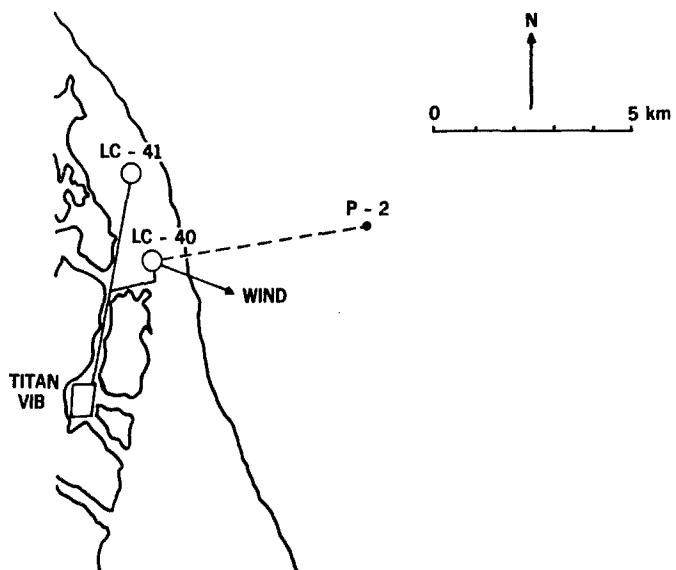


Figure 6. Sampling site of USAFSAM microcoulorometer and chemiluminescent analyzer for the 30 May 1974 launch of a Titan IIIC at Cape Canaveral AFS FL.

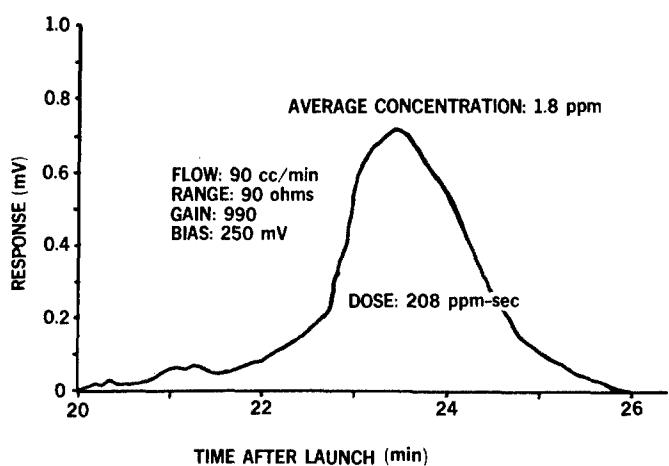


Figure 7. Microcoulometer trace of HCl detected on 30 May 1974 after the Titan IIIC launch at Cape Canaveral AFS FL.

VANDENBERG AIR FORCE BASE

Background

The USAF School of Aerospace Medicine provided instrument development and monitoring assistance to the Space and Missile Test Center in support of SAMTEC-TN-10-72-001. Our primary objective was to develop analytical tools to obtain accurate measurements of HCl in the ground cloud. Secondary objectives were to validate diffusion estimates to aid in answering biomedical questions on the potential hazard of HCl in both gas and aerosol form, and on biological synergism which might obtain if HCl and alumina particulate combined to any significant degree. A threefold instrumental approach was proposed. First, the microcoulometer was repackaged for helicopter monitoring to obtain average cloud concentration of total chloride. Second, a fast-response chemiluminescent analyzer was developed to determine cloud concentration of HCl. Third, a breadboard gas filter correlation (infrared) spectrometer was developed as an alternative instrument for measurement of gas phase HCl (Bartle, 1975). The microcoulometer and chemiluminescent analyzers were test flown in a helicopter as indicated below. The infrared detector, although conceptually adequate, proved unstable in laboratory trials and will require further development before flight test is feasible.

November, 1973

The USAFSAM participated with SAMSO/SAMTEC and the Jet Propulsion Laboratory in monitoring exhaust effluents from a Titan IIID launch at Vandenberg AFB on 10 November 1973. The USAFSAM contributed a repackaged microcoulometer adapted for continuous analysis of HCl. The sampling platform was a UHIN helicopter from the Air Force Flight Test Center, Edwards AFB, California. In successive penetrations of the ground exhaust cloud, the microcoulometer made continuous measurements of HCl concentration, in conjunction with SAMTEC deployment of pH-sensitive papers.

During the 58-minute mission, the helicopter made 20 separate penetrations of what was assumed to be the exhaust cloud. The first penetration was made at 4 minutes after launch (T+4) and the last at T+58 minutes. Figure 8 shows a computerized reproduction of the microcoulometer response as a function of time-of-flight. The numbers 1 through 20 along the abscissa

indicate cloud penetration. Table 2 summarizes the HCl dosage and average concentration determined by the microcoulometer for each of the 12 passes where positive response was obtained. Average concentration was calculated by assuming HCl was admitted only during peak (titration) rise (or the first titration rise, in the case of multiple peaks).

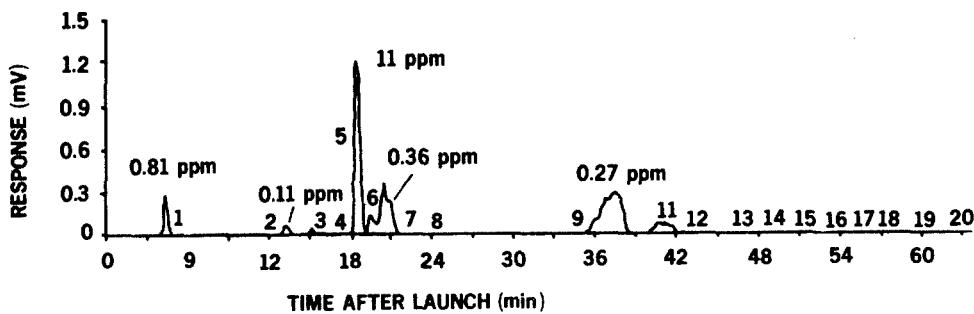


Figure 8. Coulometric airborne HCl measurements from 10 November 1973 monitoring of a Titan IIID launch, Vandenberg AFB, Calif. A computerized reproduction of the microcoulometer response as a function of time of flight.

TABLE 2. SUMMARY OF AIRBORNE HCl MEASUREMENTS
VANDENBERG AFB, CALIF., 10 NOVEMBER 1975

Peak No.	Time from Launch (min)	Altitude (ft)*	HCl Dosage (ppm-sec)	Estimated Concentration (ppm)
1	4	1700	18	0.81
2	11	1800	5.9	0.11
3	15	1400	1.3	0.11
5	18	3000	52	10.6
6-7	20	3700	67	0.36
9	35	6400	53	0.27
10-11	39	6200	11	0.17

*To convert feet to meters, multiply by 0.3048.

The maximum average concentration of 11 ppm (Table 2) was observed during pass No. 5, at T+18 minutes, and an altitude of about 3000 ft (914 m). The maximum dosage, of 67 ppm-sec, however, was observed during penetrations No. 6 and 7 at T+20 minutes. Translation of the dosage values to the ground contact/-human exposure situation requires adjustment for the relative speed of the helicopter and the prevailing wind; i.e., assuming a 3-knot wind velocity, the dosage measured at a 60-knot sampling speed must be increased by a factor of 60/3 or 20, to obtain the corresponding stationary point dosage. The concentration estimates can be translated with no adjustment. The validity of any translation is problematic, however, since the cloud was seen to rise throughout the 1-hour mission.

The microcoulometer was positioned on the left side of the helicopter behind the copilot seat. Sampling was accomplished through a 15 cm Pyrex glass tube inlet extending horizontally through the left door of the helicopter (90° to the air stream). Sample flow rate was 93 cc/min.

The results of this first monitoring test were useful to establish feasibility of the microcoulometric approach. Several operational problems were encountered during the mission which compromise the early data. First, the microcoulometer inlet line was not passivated prior to flight which may have caused sample loss due to wall absorption during the first several cloud penetrations. Second, variations in sample flow were noted during the first 10 minutes of test, but stabilized after pass 3. Third, the overcast weather conditions at launch time rendered differentiation of the exhaust cloud extremely difficult, particularly in the latter part of the mission. Certainly the data collected after T+17 minutes were from pockets of (assumed) exhaust found at altitude above 2500 ft (762 m) which was above the estimated inversion layer.

October, 1974

The second airborne monitoring test was conducted on 29 October 1974 during launch of a Titan IIID vehicle from Vandenberg Air Force Base. The equipment deployment was identical to that of November 1973. However, care was taken in this test to passivate the microcoulometer inlet by frequent injection of gaseous HCl samples up to 5 minutes before takeoff. The flight plan again called for multiple penetrations of the exhaust cloud for as long as it remained a visible entity.

The results of the October 1974 test are summarized in Table 3 by cloud penetration where visual confirmation obtained (first pass only), and by individual instrument response thereafter. The results are given for each peak in terms of HCl dosage and estimated HCl concentration. Figure 9 shows a computerized reproduction of the coulometer response as a function of flight time.

TABLE 3. SUMMARY OF AIRBORNE HCl MEASUREMENTS
VANDENBERG AFB, CALIF., 29 OCTOBER 1974

<u>Peak No.</u>	<u>Time from Launch (min)</u>	<u>Altitude (ft)*</u>	<u>HCl Dosage (ppm-sec)</u>	<u>Estimated Concentration (ppm)</u>
1	1	500	28	11
2	4	1250	350	30
3	9	3500	5	2
4	10	3500	96	19
5	10	3500	26	7
6	11	3500	30	9
7	11	3600	22	6
8	13	3700	9	0.4
9	15	4500	8	0.6
10	16	4600	29	11
11	17	4800	10	5
12	19	5400	3	1
13	24	6600	5	1
14	25	6600	2	0.2
15	28	6500	3	1
16	29	6400	3	0.2
17	38	5700	100	16
18	38	5700	78	16
19	39	5700	4	2
20	42	6000	39	1
21	44	5200	106	2
22	48	4800	28	1
23	50	4600	26	-
24	56	4300	38	8
25	56	4300	51	10
26	57	4300	15	5
27	58	4300	3	1
28	59	4300	16	3

*To convert feet to meters, multiply by 0.3048.

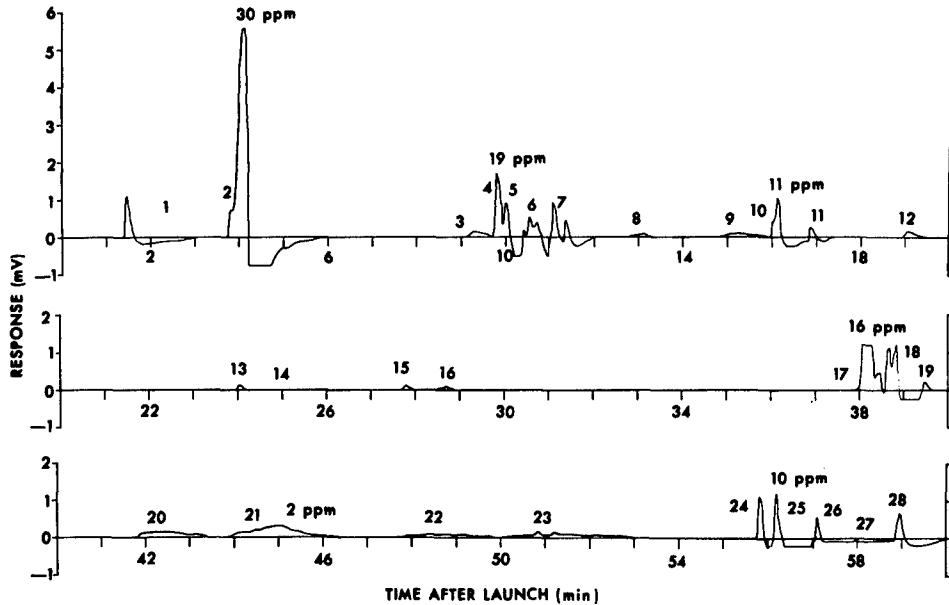


Figure 9. Coulometric airborne HCl measurements from 29 October 1974 monitoring of a Titan IIID launch, Vandenberg AFB, Calif. A computerized reproduction of the microcoulo-meter response as a function of time of flight.

The results correlate well with the November 1973 test although somewhat higher HCl concentrations were observed which prevailed for a longer period of time. However, these were mainly small tufts of exhaust found at altitudes above 3000 ft (914 m), and were probably not part of the "ground" cloud.

Pursuit of the planned mission profile was, in fact, largely negated by a fog bank which rolled in from the ocean at about T+5 minutes, and almost totally obscured the visible exhaust cloud. Hence, the decision was made, in flight, to climb above the fog bank and monitor the visible patches of exhaust.

At several times during this mission, the microcoulometer exhibited anomalous behavior. For example, the instrument gave an excessively late response to the first two exhaust cloud penetrations at T+2 and T+3 minutes respectively at 1250 ft (381 m) altitude. These two successive passes were visually well defined and noted by the flight crew, but the microcoulometer gave a single combined response (peak No. 2 in Figure 9) at about T+14 minutes. A second anomaly observed in several responses was an atypical fast decay and undershoot of the coulometric titration curve. Examples in Figure 9 include peaks 1, 6, 10, 18, and 25. Possible explanations for this behavior include power surge, intermittent electrical short circuit, and/or electrolyte displacement in the titration cell due to aircraft vibration and/or banking. The anomalies have not been successfully reproduced in the laboratory although several suggested causes have been eliminated (e.g., radio interference, large HCl dosage, and/or particulate interference). In any case, the concentration values (Table 3) for these response peaks were calculated by neglecting the undershoot area beneath the baseline, and hence may be in error.

June, 1975

A third airborne monitoring test of a Titan IIID launch was conducted at Vandenberg AFB on 8 June 1975. For this test the sampling platform was modified by the addition of a fast-response chemiluminescent HCl analyzer (Geomet Model 402B), four-channel magnetic tape recorder (Tandberg Series 115), and new sample inlet system. The magnetic tape recorder was employed to record data from the microcoulometer, chemiluminescent and (JPL) particulate analyzers, as well as a simultaneous voice record for ease of data reduction. The revised sample inlet extended out the left door of the helicopter about 4-6 in (10-15 cm) and then forward 18 in (46 cm) in the direction of flight.

The data from the 8 June 1975 launch at Vandenberg AFB are reproduced in Figure 10 and summarized on a response-by-peak basis in Table 4. Throughout this mission the correlation between visual penetration of the exhaust cloud and response of both the chemiluminescent and particulate analyzers was essentially instantaneous. Figure 11 provides a visual comparison of two one minute segments of the response from each of the analyzers on an expanded time axis. Sampling from probes on either side of the helicopter, these instruments recorded similar cloud profiles which aid in defining the cloud parameters. The overcast ceiling

1300 to 1400 ft (396 to 427 m) prevented the helicopter from penetrating the main body of the exhaust cloud during the initial, fast-rising approach to inversion layer stabilization. The overcast remained until nearly 30 minutes after launch, at which time the helicopter climbed above the natural cloud layer to search for visible patches of exhaust. Peaks labeled 1 through 13 in Table 4 were recorded below the overcast, whereas all subsequent peaks represent HCl concentrations observed in patches of brown haze found above the inversion layer. Because of baseline shift, the microcoulometer produced only four quantifiable peaks during the mission.

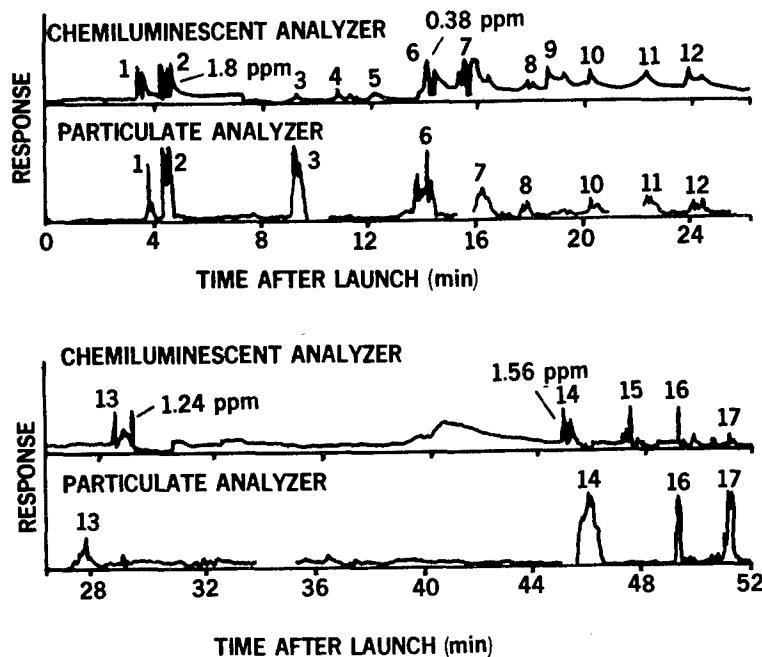


Figure 10. USAFSAM chemiluminescent HCl monitor (Geomet Model 402B) and AFJPL particulate analyzer airborne measurements from 8 June 1975 monitoring of a Titan IIID launch, Vandenberg AFB, Calif. Reproduction as taken from the Tandberg (Series 115) Instrumentation Recorder.

TABLE 4. SUMMARY OF AIRBORNE HCl MEASUREMENTS
VANDENBERG AFB, CALIF., 8 JUNE 1975

Peak No.	Time from Launch (min)	Altitude (ft)*	Microcoulorometer		Chemiluminescent Analyzer	
			HCl Dosage (ppm-sec)	Estimated Conc. (ppm)	Conc. (ppm)	HCl Dosage (ppm-sec)
1	4	1200			1.54	5.8
2	5	1200			1.80	14.6
3	9	1300			0.30	4.2
4	11	1300			0.50	5.9
5	12	1300	67.6	1.20	0.36	7.9
6	14	1300	48.1	0.87	0.38	6.5
7	16	1300	73.8	1.30	0.30	9.7
8	18	1300			0.10	1.7
9	19	1300			0.17	4.3
10	20	1300			0.15	2.5
11	22	1350	42.1	0.77	0.15	4.6
12	24	1400			0.15	5.3
13	28	1850			1.24	31.1
14	46	5300			1.56	17.8
15	48	5600			0.36	7.8
16	50	5600			0.70	6.8
17	52	5600			0.66	2.9

*To convert feet to meters, multiply by 0.0348.

AIRBORNE MEASUREMENTS OF HCl AND PARTICULATES
JUNE 1975

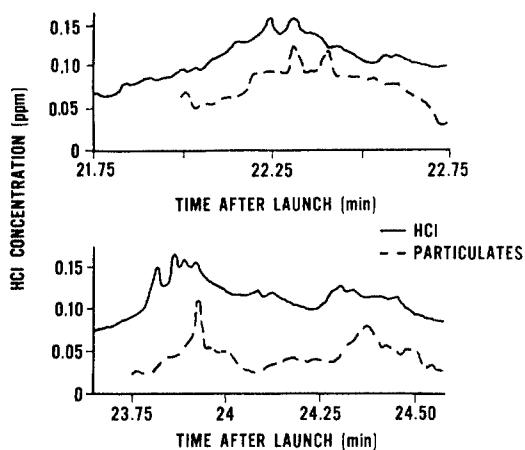


Figure 11. Airborne measurements from 8 June 1975 monitoring of a Titan IIID launch, Vandenberg AFB, Calif. Two one minute segments from the response of the chemiluminescent HCl monitor and the particle analyzer illustrating the similarities in response.

UNIVERSITY OF CALIFORNIA - RIVERSIDE

August, 1974

The USAFSAM provided HCl monitoring assistance to the University of California, Riverside (UCR), Statewide Air Pollution Research Center, in conjunction with Contract F33615-73-C-4059 with the Aerospace Medical Research Laboratory, Wright-Patterson Air Force Base, Ohio. During a 21 August 1974 visit to UCR, a USAFSAM microcoulometer was employed to determine HCl buildup and decay in experimental growth chambers used to expose ornamental plants to various concentrations of missile exhaust products. Results of these studies have been reported by Lerman et al. (1976).

September, 1975

A second-two-day series of tests was conducted at UCR on 23-24 September 1975, to validate the UCR method of analysis for HCl in plant exposure chambers. UCR employed an automatic chloride titrator to determine HCl content of bubbler samples collected in dilute nitric acid. The USAFSAM compared this method against a continuous HCl chemiluminescent analyzer (Geomet Model 401B) drawing sample directly from the plant exposure chamber.

Comparison of the measurement methods was done at five HCl concentrations in the plant exposure chamber. A schematic of the test setup is shown in Figure 12. A given concentration was obtained in the chamber by syringe pump injection of hydrochloric acid into a heated air stream which entered the chamber via an internal manifold. At each HCl concentration, chemiluminescent measurements were made by averaging three 1-minute readings, while the impinger collected a 15-, 30-, or 60-liter air sample for analysis by automatic chloride titration.

The results of the comparison are shown in Figure 13. Agreement between the two methods was within the limits of experimental error. The chemiluminescent analyzer was prestandardized against the chloride titrator using a steady but unknown concentration of HCl in nitrogen supplied by an oven calibration system (A.I.D. Model 309). Four calibration runs were made during the two days of experiments. With the same flow conditions on the calibration delivery oven, the chemiluminescent analyzer gave a response of 3.4 ± 0.3 volts to a titrator measured concentration of 4.2 ± 1.2 ppm (by volume) HCl (Table 5).

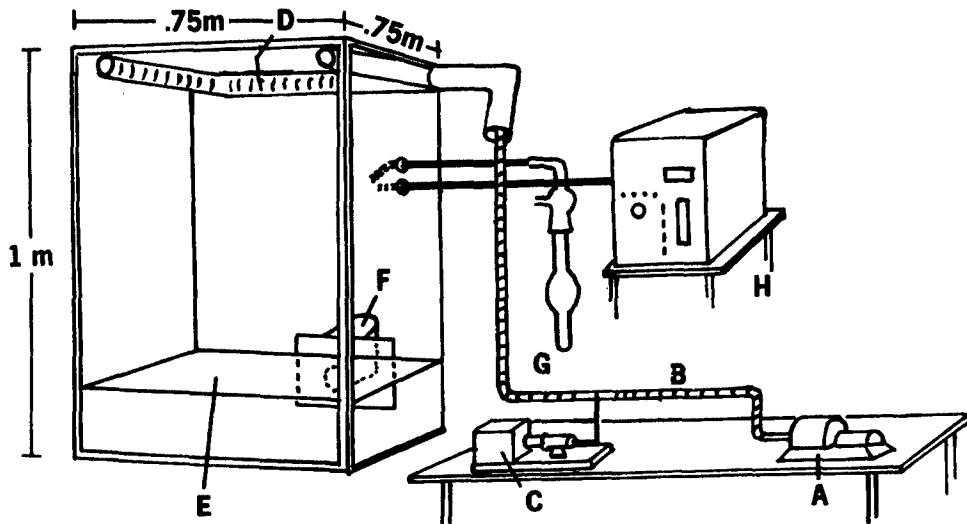


Figure 12. UCR plant exposure chamber. A. compressor; B. heated line; C. syringe pump for HCl injection; D. dispersion manifold; E. perforated base; F. high-volume exhaust motor; G. bubbler for HCl collection in dilute nitric acid; H. chemiluminescent HCl monitor.

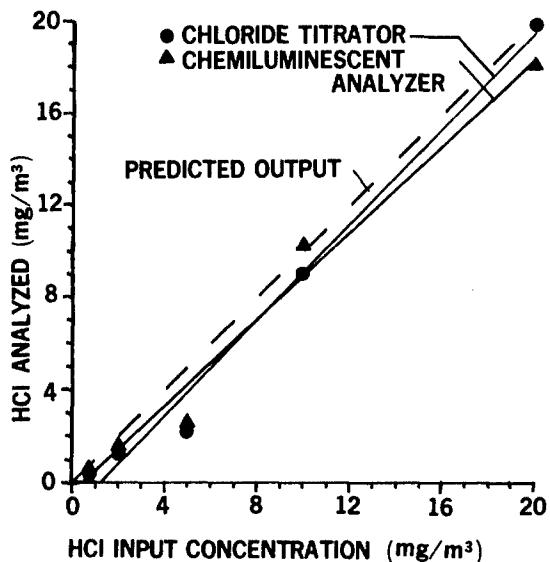


Figure 13. UCR chloride titrator vs chemiluminescent HCl monitor (Geomet Model 401B). Data verifying the chloride titrator (HCl) detection procedures in the plant exposure chambers.

TABLE 5. CHEMILUMINESCENT HCl ANALYZER CALIBRATION DATA,
UNIVERSITY OF CALIFORNIA, RIVERSIDE, CALIF.,
23-24 SEPTEMBER 1975

<u>Date/Time</u>	<u>CHEMILUMINESCENT Analyzer Response (Volts)</u>	<u>Chloride Titrator Concentration</u>	
	<u>ppm</u>	<u>mg/m³</u>	
23 Sept/morning	3.25	2.6	4.2
23 Sept/afternoon	3.10	5.2	8.4
24 Sept/morning	3.80	3.9	6.3
24 Sept/afternoon	3.30	5.2	8.5
Average	<u>3.4±0.3</u>	<u>4.2±1.2</u>	<u>6.9±2.0</u>

DISCUSSION AND CONCLUSIONS

The field test program to date has provided a clear indication of instrumentation preference for specific applications, but only preliminary insight into the behavior of the exhaust cloud following rocket launch. The detection concept and fast-response characteristics of the chemiluminescent analyzer make it clearly superior for the airborne-monitoring application. The microcoulometer by contrast has proved itself as a versatile laboratory tool and a useful field instrument for HCl detection in ground-monitoring applications. The fact that the microcoulometer is a primary standard for total chloride analysis alleviates the time consuming and difficult task of field calibration. The principal disadvantages of the microcoulometer are its slow time of response and somewhat limited dynamic range, which are of minor concern in the low-level relatively slow exposure situation encountered in ground monitoring. In comparison, the chemiluminescent analyzer gives an instantaneous response to gaseous HCl which is highly advantageous for airborne measurement. The chief disadvantage of the chemiluminescent approach is that it is not a standard for measurement of HCl gas but must be calibrated with a known concentration or referee method. This disadvantage is partially compensated by the fact that the chemiluminescent response is linear with HCl concentration which normally permits single-point calibration. Where logistics allow, the use of both instruments is advantageous since the microcoulometer measures total chloride and the chemiluminescent analyzer is specific for gaseous HCl.

With respect to cloud behavior, the USAF data base is somewhat meager and subject to conflicting interpretation. On the one hand, none of the airborne or downwind surface levels have exceeded the short-term public limit of 4 ppm for 10 minutes recommended by the National Academy of Sciences. Overall, the measured HCl concentrations have tended to be lower than predicted by diffusion modeling. This finding is an essential agreement with the more extensive results from the NASA program at Cape Canaveral (Gregory et al., 1976). Translation of Eastern Test Range data to Vandenberg AFB, however, must be done with due consideration of the climatic and topographic differences. On the other hand, instantaneous concentrations at or above 4 ppm have been measured as long as 50 to 60 minutes after launch at altitudes above the inversion layer, 5-15 miles (8-24 km) from the launch site. The emerging picture on exhaust cloud behavior at Vandenberg is one of generalized downwind drift with slow disintegration into segments, rather than bulk diffusion of a single entity. The cloud segments, although individually small, may contain relatively high concentrations of HCl in gaseous form. However, these high measurements have been predominantly above the inversion layer. The data collectively neither support nor refute the return of the "ground" cloud to the surface, as predicted or assumed in diffusion modeling.

In terms of data collection, there appears little doubt that airborne monitoring has been more cost effective than ground measurements at Cape Canaveral. Although the prevailing overcast at Vandenberg AFB has, in part, compromised each mission, the mobile sampling platform nonetheless provides opportunity for multiple penetrations of the exhaust cloud and thus greatly increased chance for data collection. The use of helicopter side inlets for airborne sampling is somewhat problematic because of the adverse influence of rotor turbulence. Sampling of the undisturbed cloud can be done more effectively with nose-mounted inlet probes on either a helicopter or small fixed-wing aircraft. In a joint sampling effort with the Environmental Protection Agency, we observed excellent correlation between the microcoulometer and chemiluminescent analyzers using nose sampling from a Beech C-45 aircraft to monitor the exhaust plume from the incinerator ship Vulcanus (Ligday and Giannetta, 1975).

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OVERVIEW OF HYDROGEN CHLORIDE AND CHLORINE
AS PHYTOTOXICANTS

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Hydrogen chloride (HCl) was discovered in the 15th century and during succeeding centuries its properties were described. The phytotoxic properties of HCl began to receive considerable public attention during the 19th century. The first large scale production of HCl reported in the literature was the development of the Le Blanc process for the production of soda ash. In this process salt and sulfuric acid were reacted to produce salt cake; the HCl byproduct was vented to the atmosphere. Extensive vegetation injury from the HCl occurred in areas adjacent to the soda ash plants in England and continental Europe.

Haselhoff and Lindau (1903) published a review chapter describing events related to the development of an HCl air pollutant problem and regulations to limit discharge to the atmosphere. The Lord Derby Alkali Act enacted in Great Britain in 1863 required manufacturers to limit releases of HCl to 5% of the total produced in their operations. Subsequently, scrubbing techniques were developed to recover the gaseous HCl. This technique was so efficient that 1,181 plants in Great Britain in 1900 were recovering 98.55% of the HCl that was formerly released to the atmosphere. Industrial uses for this acid byproduct developed rapidly and soon HCl became the prime product and soda ash was of secondary importance.

Industrial use of HCl and Cl₂ has increased rapidly during the past 50 years. Production of HCl in the United States was 63,000 tons in 1933 and increased to 2 million tons from 150 facilities in 1975. Ninety percent of this production is a byproduct in the chlorination of hydrocarbons for the manufacture

of pesticides, plastics and aerosol propellants. While chemical plants are the major source of HCl, it is also produced by combustion of fossil and rocket fuels. Incineration of municipal refuse and agricultural waste can contribute significantly to HCl released to the atmosphere. Salt in human food and chlorides in plant and animal tissues are converted to HCl upon incineration of the materials. Such sources are generally insignificant but HCl injury has been reported on plants growing in close proximity of incinerators where large volumes of trash were burned.

Chlorine (Cl_2) is widely used commercially because of its strong oxidizing property. About 10 million tons of this gas are produced annually in the U.S. and most of it is used by the chemical industry. It frequently appears in combination with HCl in polluted atmospheres but vegetation injury usually results from accidental releases. It is necessary to transport large quantities of both Cl_2 and HCl since the site of their production is often some distance from the point where they are to be used. Human error, failure of equipment and accidents during transport, transfer and handling of these compounds sometimes result in short-term massive releases of the gases. Under such conditions severe injury to vegetation, animals, and materials may occur in a limited area.

In 1961 a tank car containing 30 tons of chlorine derailed and spilled in a rural community (Jayner and Durel, 1962), and the gas spread over 20 square miles. Injury symptoms similar to those produced by sulfur dioxide developed on vegetation and there was widespread injury to livestock. Pregnant cows aborted, chickens stopped laying and premature molting was initiated. Some chickens laid eggs without shells and the yolks were congealed.

One of the earliest reports of chlorine injury to vegetation in the U.S. was by Stout (1932) where a spill occurred during the chlorination of sewage. Lettuce and weeds growing nearby were injured. With the increased use and transport of chlorine during the succeeding years, incidents of accidental spills resulting in severe vegetation injury in localized areas have increased markedly.

SYMPTOMATOLOGY

Chlorine, an essential nutrient element for higher plants, is toxic when accumulated in excessive amounts or when plant foliage is exposed to excessively high concentrations of Cl_2 or HCl gas. Injury symptoms from soil, water or air exposure to these compounds may cause a broad range of responses including: bronzing and greying of leaf surface, chlorosis at the apex, margin or base of the leaf; necrosis at the apex, margin and between the major veins; distortion or curl of leaves; and premature and excessive abscission of foliage. Plant growth suppression may occur with chloride accumulations well below levels required to produce leaf symptoms.

The most common acute injury symptom observed on broad-leaved plants is the marginal or intercostal necrosis. The necrotic tissue may be white, yellowish-brown, brown, red or nearly black in color. Frequently leaves of almond, tomato, bean, zinnia, etc. may develop glazing or a grey discoloration of the leaf surface. The glazing may develop into a bronze discoloration with time. Haselhoff and Lindau (1903) described symptoms of acute injury from HCl on a variety of plants. Leaves of grape vines exhibited red edges and dahlias developed black edges. The margins of pear leaves were nearly black and sometimes the entire leaf became black. The dark discoloration was thought to be due to deposits of dark tannin substances. Roses were observed to develop dark colored spots and cells in these spots were filled with a black substance.

The necrotic lesions on broad-leaved plants may be surrounded by a zone of bleached tissue. Irregular shaped strips of necrotic tissue may extend between the major veins inward from the leaf margin toward the midrib. With leaves such as almond these necrotic strips may break away, leaving a ragged, feather-like leaf appearance.

Chlorine injury on dicots (broad-leaf plants) frequently appears first as a cooked (very dark) green area that later turns brown. These spots are sometimes found between the veins, but they are usually concentrated near the leaf margin (Hicks, 1976). For the most part, injury symptoms from Cl_2 are very similar to those produced by HCl.

Injury symptoms on grasses (monocots) exposed to HCl or Cl₂ usually develop first as dark green (watersoaked) streaks between the major veins. These streaks are concentrated in the region between the tip and point where the leaf bends down. With time, the streaks become chlorotic (yellow) or straw brown necrotic lesions. With repeated exposure to chloride, grass leaves may develop a marked zone of necrotic tissue at the leaf tip and along the leaf margin near the tip.

General chlorosis, marginal chlorosis and/or irregular strips of chlorotic tissue between the major veins of dicots may develop on plants exposed to dosages of HCl or Cl₂ which are insufficient to produce necrotic lesions.

HCl injury on needles of mugo pine developed as pale discolorations with a browning and necrotic appearance spreading from the tip (Haselhoff and Lindau, 1903). Examination of needle sections revealed that grains of chlorophyll were bleached and were no longer clearly recognizable. Fumigation with chlorine produced tan, brown or reddish necrosis which extended toward the needle base with increased severity of exposure (Hicks, 1976). Mottling of the needle may occur also.

Accumulation of chloride from chronic exposure to HCl and chlorine may induce chlorosis generally on the younger, developing leaves. The leaves may also develop an upward curling and leaf abscission may be stimulated. Observations in the field following accidental releases of chlorine have revealed rapid abscission of leaves on eucalyptus, elm and fig trees. The brief exposure to a very high concentration cloud of chlorine resulted in almost complete defoliation of mature trees within 8 to 10 hours.

Weiler (1934) reported extensive damage to plants in the form of dots and spots on the leaves if rain, dew or fog served as transmitters for acid in the atmosphere. He pointed out that the best known type of damage caused by hydrochloric acid is the peculiar ring formation on leaves which was shown by Schroeder and Reuss. Weiler (1934) described the ring-like spots observed on vegetation in the field and concluded that the wide distribution of injury around the industrial source occurred because the acid was carried along by soot particles or droplets of fog.

Weiler (1934) observed white spots on sugar beets and concluded that they were the result of chlorophyll destruction by chlorine gas. He pointed out that the leaves have stomata on the upper side through which the chlorine apparently entered. He also described in some detail anatomical changes which accompanied injury induced by acid forming gases in polluted atmospheres.

CHLORIDE ACCUMULATION

Haselhoff and Lindau (1903) concluded that HCl would react with alkaline materials in the soil and that the dissolved salts would be expected to leach through to the subsoil. It was assumed that the amount of Cl₂ and HCl released by industries prior to their publication was too small to affect soil composition and substantially interfere with plant growth. Possible pH change, deposit of chlorides or nutrient alteration induced by massive release of Cl₂ and/or HCl over a limited area have not been adequately investigated. The possible impact of acid precipitation or ground level reaction of gaseous HCl and Cl₂ on soil solutions is not known. Chloride salts are injurious to plants when a critical level is exceeded but in some instances seed germination and plant growth are enhanced (Haselhoff and Lindau, 1903; Lind and Landon, 1971).

Chlorine is absorbed as a chloride ion from soil media and is transported to actively growing parts of the plant. When low levels of chloride are applied in nutrient solution, the chloride accumulation appears to be relatively uniform throughout the plant, but when high concentrations are applied the older leaves accumulate the highest concentrations. Chloride is readily absorbed through the leaves when irrigation water with high salt content is deposited on them by sprinklers. Severe leaf injury may result from such deposits. Excessive accumulation of chloride may occur when foliage is exposed for long periods to sub-lethal concentrations of Cl₂ and HCl in the air. In general, marginal chlorosis and/or necrosis can be expected when chlorine ion levels from long-term accumulation approaches 2 percent on a dry leaf tissue basis. Severe injury to leaf tissue may occur without measurable increase in chloride content when plants are exposed to very high concentrations of Cl₂ or HCl for a few minutes (Brennan et al., 1965).

TABLE 1. RELATIVE SUSCEPTIBILITY OF PLANTS
TO CHLORINE (HICKS, 1976)

<u>Susceptible</u>	<u>Intermediate</u>	<u>Tolerant</u>
Alfalfa	Annual blue grass	Azalea
Blackberry	Buckwheat	Balsam fir
Box elder	Cheeseweed	Begonia
Chickweed	Corn	Cactus
Crab apple	Cucumber	Chenopodium
Gomphrena	Dahlia	Chrysanthemum
Horsechestnut	Dandelion	Cowpea
Mustard	Nasturtium	Field corn
Pin oak	Nettle-leaf goosefoot	Geranium
Radish	Onion	Kentucky bluegrass
Rose	Petunia	Lamb's quarter
Sugar maple	Pinto bean	Mignonette
Sunflower	Scotia bean	Myrtle
Sweet gum	Squash	Oxalis
Tobacco	Tomato	Pepper
Tree-of-heaven		Pigweek
Tulip		Pine
Virginia creeper		Polygonum
Wandering Jew		
Zinnia		

TABLE 2. RELATIVE SUSCEPTIBILITY OF PLANTS
TO HYDROGEN FLUORIDE (HICKS, 1976)

<u>Susceptible</u>	<u>Intermediate</u>	<u>Tolerant</u>
Caespitose phlox	Black cherry	Adonis
Columbine	Chrysanthemum	Arborvitae
Cornflowers	European black alder	Austrian pine
Garden daisies	Norway maple	Balsam fir
Oriental poppy	Sugar maple	Douglas fir
Tomato	Pinto bean	Garden iris
Tulip tree	White pine	Garden lupin
		Garden peony
		Goldenrod
		Larkspur
		Lily-of-the-valley
		Norway spruce
		Paniculate phlox
		Plantain lily
		Pheasant's eye pink
		Red oak
		Solidago
		Sweet William

MATERIALS

Chlorine and HCl gas discharged into the atmosphere are highly soluble in suspended moisture or condensed droplets on surfaces. The acid produced is corrosive and may cause significant damage to manufactured products. Excessive corrosion of security fences and metal structures is frequently observed in areas adjacent to industries where Cl₂ and HCl is released intermittently. Corrosion of aluminum window frames and hardware on residences has been observed where HCl was released to the atmosphere. Because of the solubility of Cl₂ and HCl, acid aerosol formation can be expected when these gases are discharged into moist atmosphere and corrosion of exposed metals is inevitable.

CONCLUSIONS

Phytotoxicity of HCl was recognized well over a century ago, but because of the efficiency of scrubbing systems developed to meet air quality regulations the air pollutant threat was minimized for about 50 years. Rapid expansion in manufacture and utilization of Cl₂ and HCl for production of pesticides, plastics, refrigerants, aerosol propellants, paper, and rocket fuels has increased the environmental threat from these materials in certain localized areas. Phytotoxicity and damage to materials is seldom a problem unless massive releases occur from accidental spills or combustion of high chlorine content fuels.

Phytotoxicity of Cl₂ and HCl is affected by light intensity, relative humidity, species and variety of plant exposed, physiological age of leaf tissue, concentration of the gas, and duration of the exposure. Other environmental factors as well as interactions of those listed are probably responsible for the reported differences in dosages required for injury. Haselhoff and Lindau (1903) reported that 1500 mg HCl/m³ for one hour was required to produce lesions on fir, beech, and oak and 3000 mg HCl/m³ for one hour daily for 80 days caused marginal necrosis on leaves of maple, birch, and pear. Means and Lacasse (1969) found that tree species could be injured with 3 to 43 ppm HCl

in a 4 hour exposure. Lind and London (1971) found that 95 ppm of HCl produced slight injury on marigold and 300 ppm produced severe injury but not death. Lerman et al. (1975) reported injury on several flower species when they were exposed to HCl concentration ranging from 1.5 to 35 mg HCl/m³ for 20 minutes.

To assess damage caused by HCl or Cl₂ exposures in the field it is essential that as many environmental conditions as possible be considered. Accuracy of such assessments will probably be enhanced markedly when dose response investigation can be conducted at least with major species in the area impacted.

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GENERATION OF HCl GAS AND Al₂O₃ PARTICLES FOR THE EXPOSURE
OF EXPERIMENTAL PLANTS UNDER CONTROLLED CONDITIONS

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INTRODUCTION

Evaluation of the phytotoxic effects of rocket exhaust products on plants depends upon the ability to generate controlled quantities of these combustion products, either individually or in combinations. Solid fuel motors planned for use in the space shuttle program produce four major exhaust components: 31% CO₂, 22% Al₂O₃, 21% H₂O, and 24% HCl on a weight percent basis (Goldford, 1976). Three LO₂-LH₂ engines burning in parallel will also contribute large amounts of H₂O which may condense on the Al₂O₃ and result in an adsorbed HCl ("Space Shuttle Exhaust Effects on Biota," 1973) film (Environmental Statement USAF Space Launch Vehicles, 1975). Of these products, aluminum oxide and hydrogen chloride present a potential pollution problem. The plant community surrounding a launch site may be exposed to a large variation in pollutant cloud dispersion (Environmental Statement USAF Space Launch Vehicles, 1975). Modified equipment assembled for laboratory studies as described herein allows improved simulations of potential field emissions.

Initial experimental work (Lerman, 1974; Lerman, 1975) measured the response of several ornamental plant species to short duration, 20 minute, HCl exposures. In additional work, HCl gas and aluminum oxide particulates were combined in a 1:1 ratio (Lerman, 1975). The HCl gas was generated by a method described by Hill et al., (1959) in which a metered air flow

was bubbled through a heated aqueous solution of HCl and entered into the exposure chamber air flow. It was necessary to make daily calibration curves because the aqueous HCl changed concentration as it became depleted. Satisfactory prediction of the plant exposure chamber concentration proved difficult if several fumigations were made during one day. The current method described here features a standard calibration curve which is independent of the number of fumigations performed.

The Al_2O_3 aerosols exiting a solid rocket motor have been characterized as being less than one micron in diameter (Lerman, 1975). The assumption is made that under the concentrations present in an exhaust plume, these particles will rapidly agglomerate forming a particle size distribution with a mean size of 10 microns (Environmental Statement USAF Space Launch Vehicles, 1975). Aerosols generated for experimental fumigations should also have a large submicron fraction when initially combined with HCl gas in order to simulate the increased surface area present for adsorption.

Aluminum oxide particles were originally introduced through a vibrated hopper, brush-fed system adapted from a Bahco micro-particle classifier. To control particle size (Lerman, 1975), particles entering the air stream travelled through a modified glass cyclone and then through a single stage slotted impactor. This method of Al_2O_3 aerosol generation relied on uniformly dispensing a preweighed amount of the dust over the duration of the fumigation. The system delivered particles of a uniform size distribution, but required operator attention before, after, and during each fumigation. Newly designed equipment can be operated for over four hours without refilling. A standard calibration curve enables the operator to preset the delivery concentration.

The methods described in this paper represent relatively inexpensive and simple means for the controlled production of HCl gas and Al_2O_3 particulates. Both pollutants can be generated over a wide range of concentrations without requiring attention during a fumigation.

MATERIALS AND METHODS

HCl GAS GENERATION

Gaseous HCl is generated using a motorized syringe pump to inject aqueous HCl into a heated air stream for vaporization. The final HCl(g) concentrations can be varied by changing either the injection rate and/or the concentration of the HCl acid injected. This method is similar to that described by Mandl et al. (1971) for the generation of HF gas.

A Sage model 351 syringe pump was selected because of its capability to dispense two syringes simultaneously and for its wide range of delivery rates (0.016 to 60 ml/min). One of three infusion rates (0.17, 0.13 or 0.084 ml/min) is normally used for fumigations. The solutions were prepared using double distilled water and analytical grade hydrochloric acid (37% HCl by weight).

A Hamilton three-way inert valve allows a 10 ml syringe to be filled and emptied without removal from the Sage pump. When the valve is set for injection, the acid is carried into and through one arm of the tee fitting by a 20 gauge teflon needle (Figure 1).

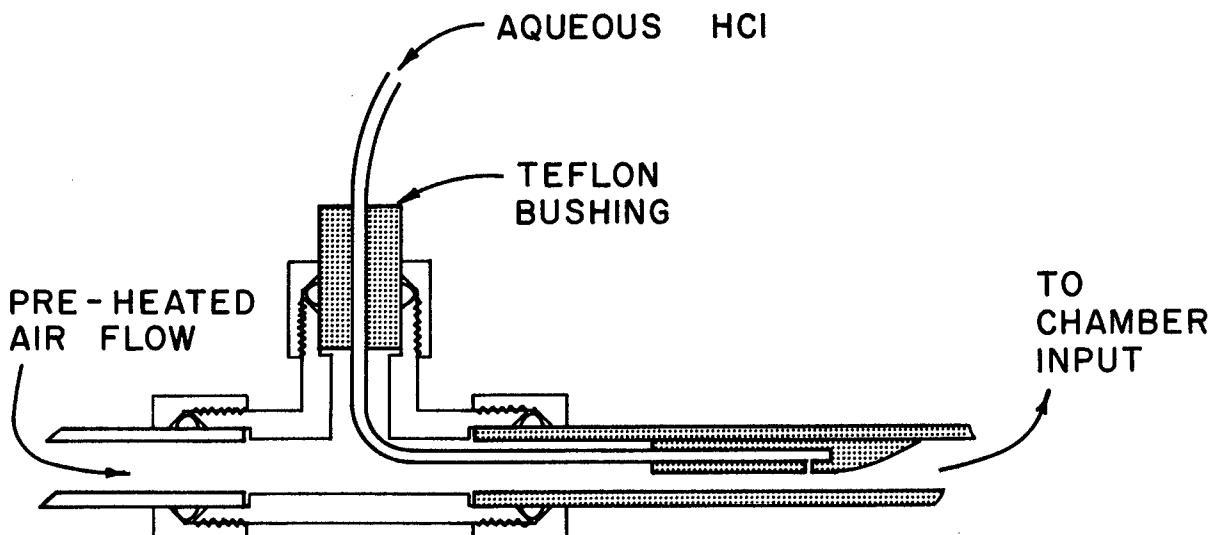


Figure 1. Tee fitting joining the 3/8" O.D. copper tubing, 20 gauge teflon needle and 3/8" O.D. teflon tubing.

Five feet of copper tubing, wrapped with a heat tape and insulation, is used to preheat a 12 liter/minute air stream to 90 C before it passes through the tee fitting. The warm air stream joins the volatilizing acid at one end of a four-foot length of 3/8" O.D. teflon tubing heated to 105 C. The tube, in turn, delivers the volatilized HCl to the exposure chamber input manifold (Figure 2). Chamber concentrations were measured using a 0.1N nitric acid scrubber and/or a Geomet chemiluminescence HCl(g) monitor.

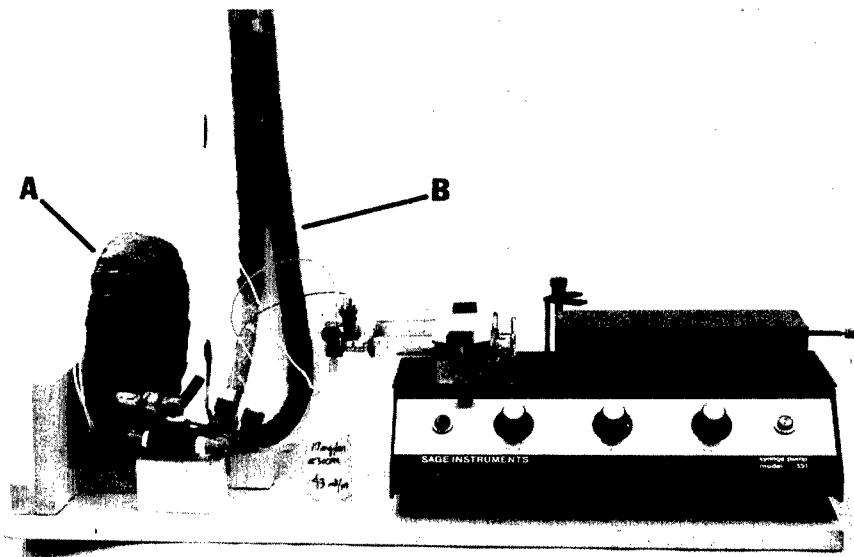


Figure 2. HCl(g) generation system for simultaneous infusion with two syringes. A. Five feet of heated copper tubing. B. Four-foot lengths of teflon tubing. Heat tape partially exposed on left tube.

Al_2O_3 AEROSOL GENERATION

Aluminum oxide aerosols are generated and sized by combining a fluidized bed generator and a vertical settling chamber (Figure 3). Dry nitrogen passing through the Al_2O_3 bed entrains the particles and moves them through the generator. This delivery concentration (mg/m^3) is varied by changing the N_2 flow rate, and is restricted by the nominal settling velocity of the upper particle size limit chosen.

TO EXPOSURE CHAMBER

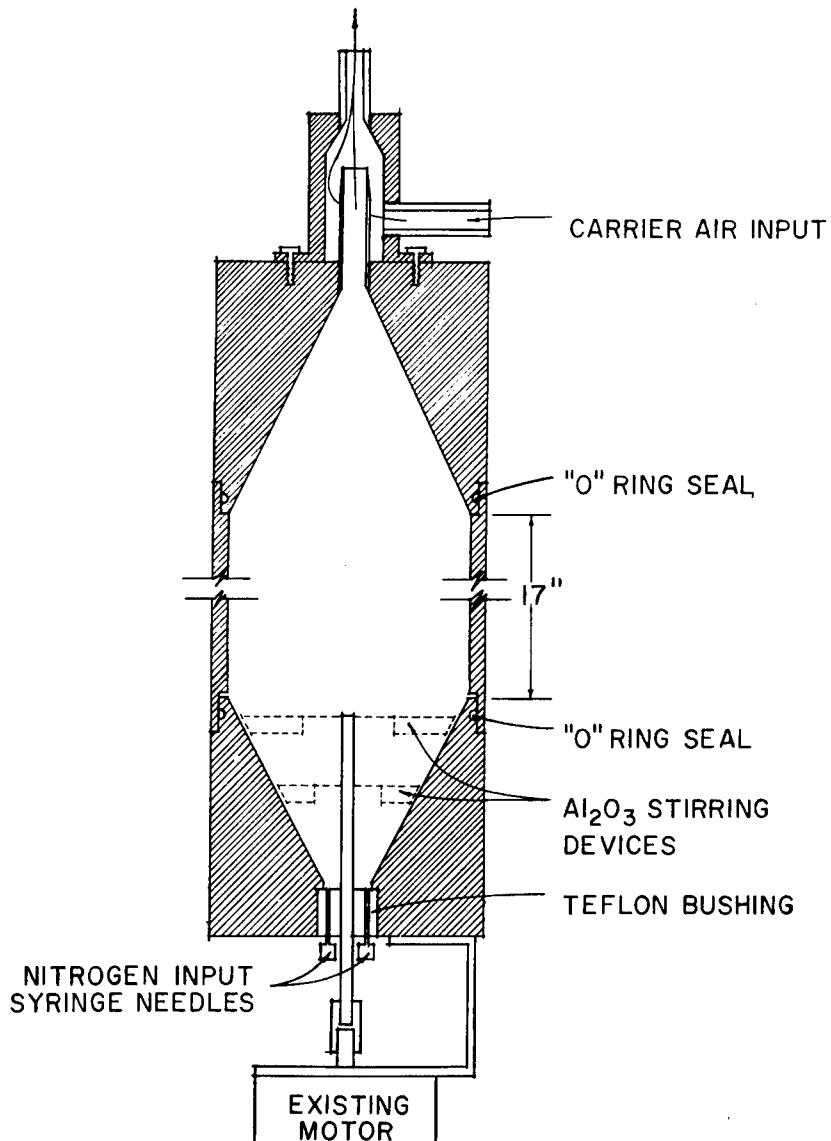


Figure 3.
Aluminum oxide
aerosol genera-
tor. Machined
from aluminum
stock.

A 4-inch I.D. aluminum tube was used in construction, with "O" ring-sealed-end caps milled from solid aluminum. All internal surfaces were highly polished to reduce turbulence. The flow-metered N₂ passes through three 27-gauge needles to the aluminum oxide reservoir, where wire configurations and paddles turning at one RPS keep the dust fluidized and free from channeling. Particles exiting the top of the generator are immediately diluted 2:1 by a 4-liter/minute air stream and moved rapidly into the chamber input manifold.

The submicron aluminum oxide particles were the same as previously described by Lerman (1974). A scanning electron micrograph (Figure 4) shows the characteristic texture.

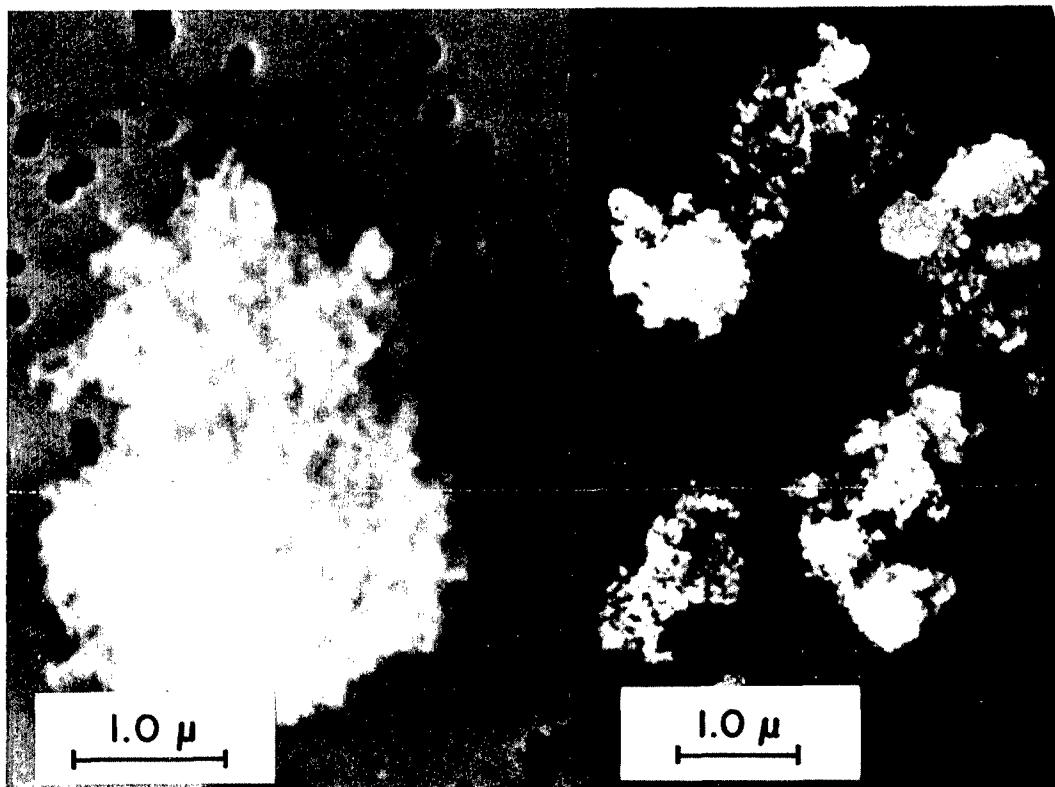


Figure 4. Scanning electron micrograph showing characteristic texture of Al_2O_3 particles. LEFT: particle impacted on $0.2 \mu\text{m}$ nucleopore, 25KV, 10,000X. RIGHT: particles impacted on aluminum foil, 25KV, 10,000X.

Particle size distributions were determined at the point of entry into the chamber using a Weathermeasure high volume cascade impactor and a modified Bausch & Lomb 40-1 optical particle counter. Gelman glass fiber filters, type AE, were used for all impaction and collection surfaces. The collection efficiency curves and particle cutoff sizes for the cascade impactor were adjusted for the true density of aluminum oxide (3.7 gm/cm^3). Calibration of the optical counter was performed using mono-disperse polystyrene latex (PSL) beads. The refractive index of PSL is 1.6 which is close to that of gamma aluminum oxide, 1.7.

EXPOSURE CHAMBERS

Figures 5 and 6 illustrate the aerosol fumigation system. The exposure chambers used are similar to that described by Heck et al. (1968). The aerosol chamber has an input blower maintaining 1/4" H₂O negative pressure during fumigation. Further modification allows the doors to be raised on a counter-weight system, enabling insertion of plants and reclosure in less than 15 seconds. The gas and aerosol distribution within these chambers has been measured and reported previously (Lerman, 1974).

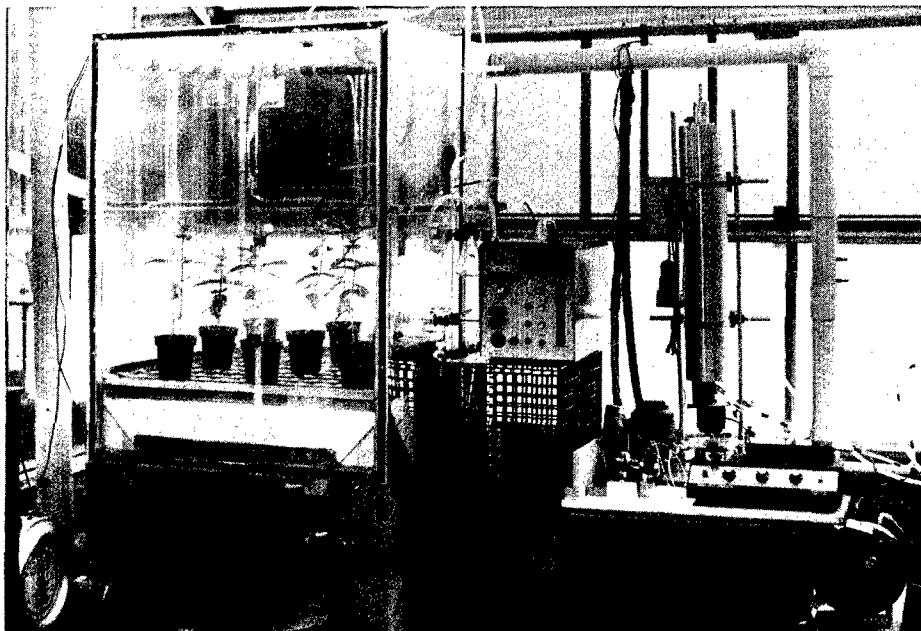


Figure 5. Plant exposure chamber for gas/aerosol studies.

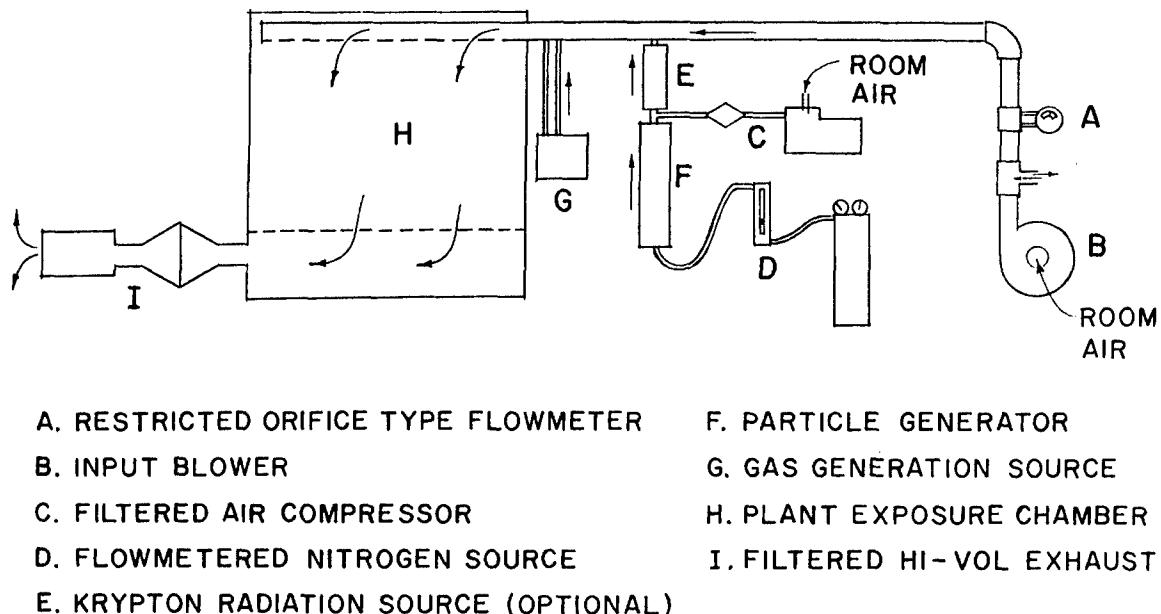


Figure 6. Schematic diagram of the gas-aerosol exposure chamber system.

RESULTS AND DISCUSSION

VAPORIZED HCl GENERATION

The standard calibration curves (Figure 7) for one of the three HCl fumigation chambers represent the calibration of an empty chamber and of one containing a full load of 16 mature marigold plants. The biomass of plants to be fumigated must be considered prior to selecting the HCl concentration for injection. The lower curve in Figure 7 is only valid for the number, leaf area, and species of plant used, yet it serves as a guide for estimating the effect of plants as a HCl gas sink. For example, using the plant load curve of Figure 7, injecting a solution containing 160 mg HCl/ml would yield a 17 mg HCl/m³ chamber

concentration. On the other hand, the same solution would give an empty chamber concentration of 26 mg HCl/m³. Figure 8 is a constant monitor trace of an actual 20-minute fumigation of 6 bean plants. The trace of exposure chamber concentration demonstrates the immediate drop for 20 mg/m³ to 17 mg/m³ after the addition of the 6 plants. The amount of time required for the chamber concentration to reach equilibrium averages one minute, with only 30 seconds being required to obtain 90% of the final concentration.

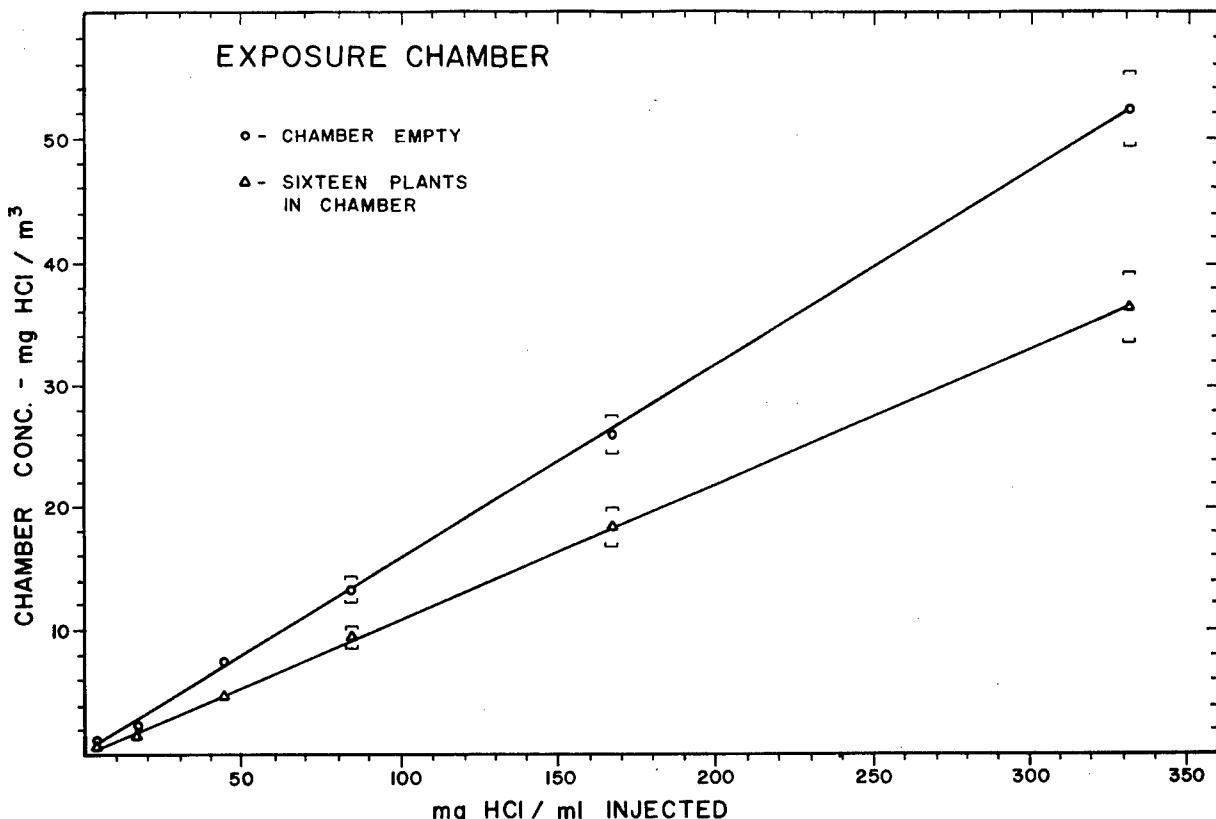


Figure 7. Standard calibration curves for volatilized hydrogen chloride gas with and without a plant load. Six replications per point.

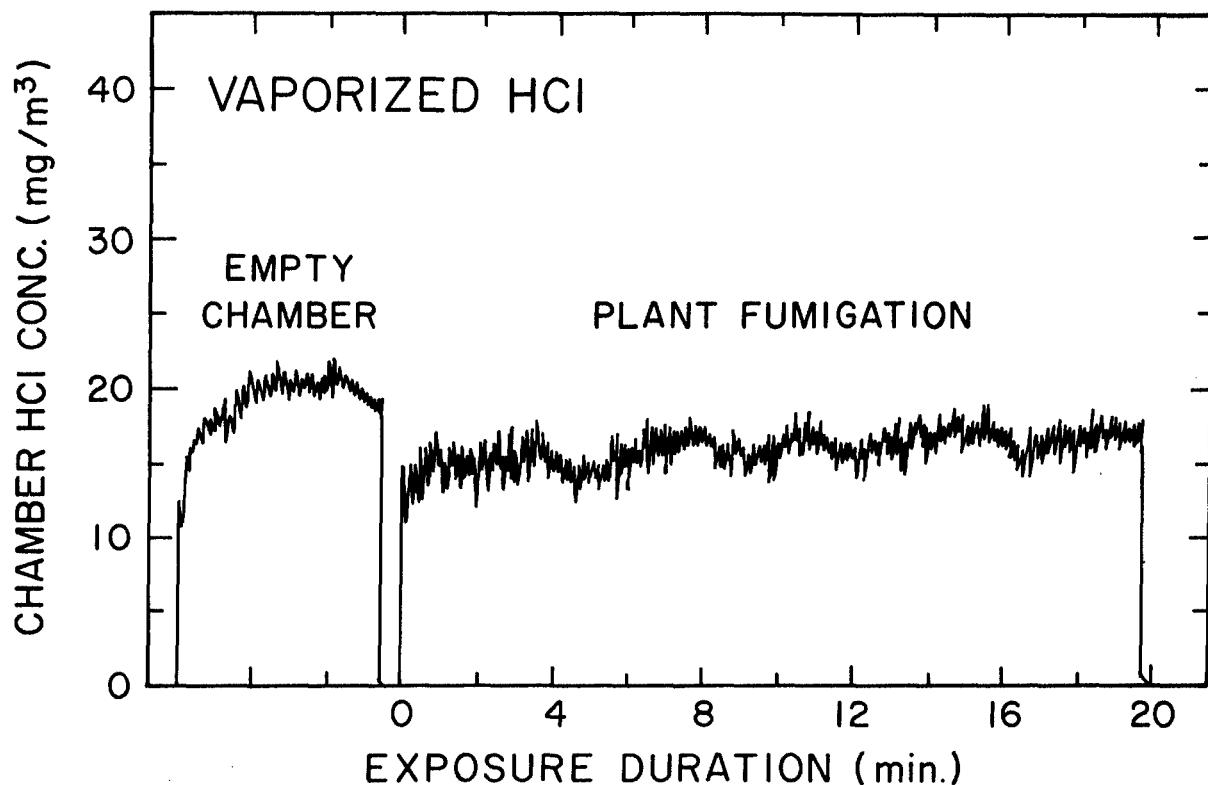


Figure 8. A recorder trace of a 20 minute HCl gas fumigation showing the drop in empty chamber concentration caused by 6 bean plants.

Al₂O₃ AEROSOL GENERATION

The calibration curve for the aerosol generator (Figure 9) represents fumigation with a 30 CFM (0.849 m³/min) air flow through the chamber. The apparent linear response of the generator output to increases in N₂ flow rate has only been defined between 0.75 and 1.3 liters/minute. Further calibration work will characterize the generator response between 1.3 and 2.0 liters N₂/minute.

Size distributions of the alumina particles have been plotted logarithmically in Figures 10 and 11. The mean particle diameter, as measured by either % number or % mass, increases as the nitrogen flow rate through the generator is increased (Figure 10). This change in distribution was anticipated and is small

enough to be acceptable for the generator's application. Shifts in the % number particle size distribution during 3.5 hours of operation (Figure 11) are also considered acceptable for fumigation work. The mean particle diameter shifts from 1.4 μm to 1.7 μm during the first two hours of operation and then appears to stabilize for the remaining 1.25 hours. When studied together it is felt that Figures 10 and 11 show that the Al_2O_3 generator is capable of delivering a relatively stable size distribution for extended periods of time.

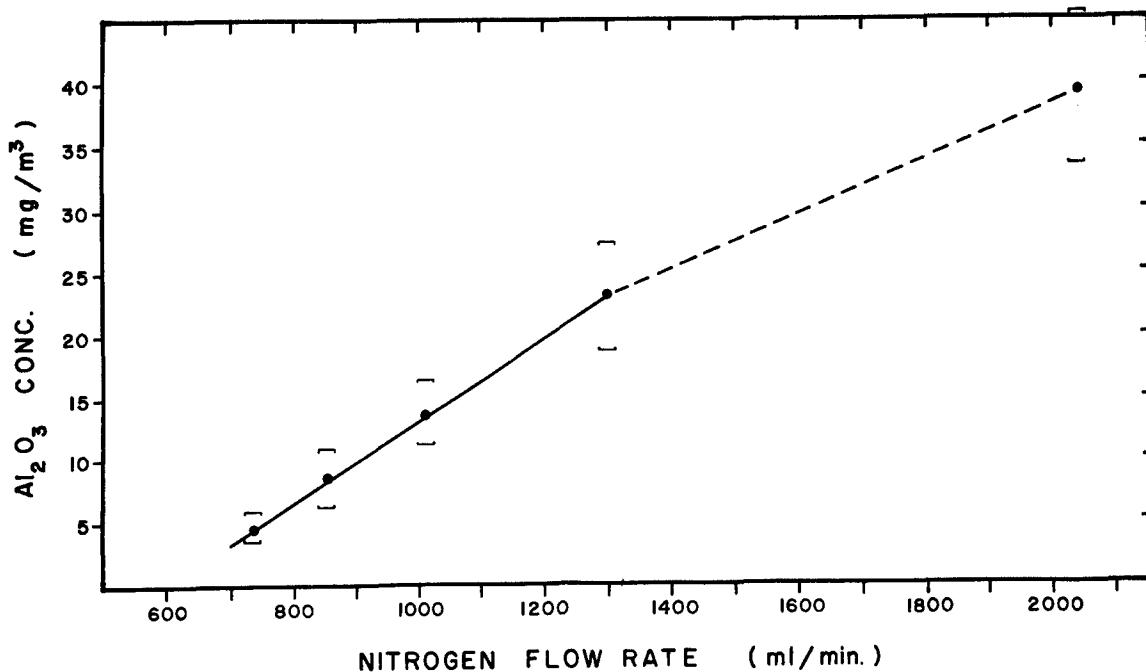


Figure 9. Standard calibration curve for aluminum oxide particulate generator. The dashed line represents the portion of the generator response not defined. Brackets refer to one standard deviation about a mean of four replications.

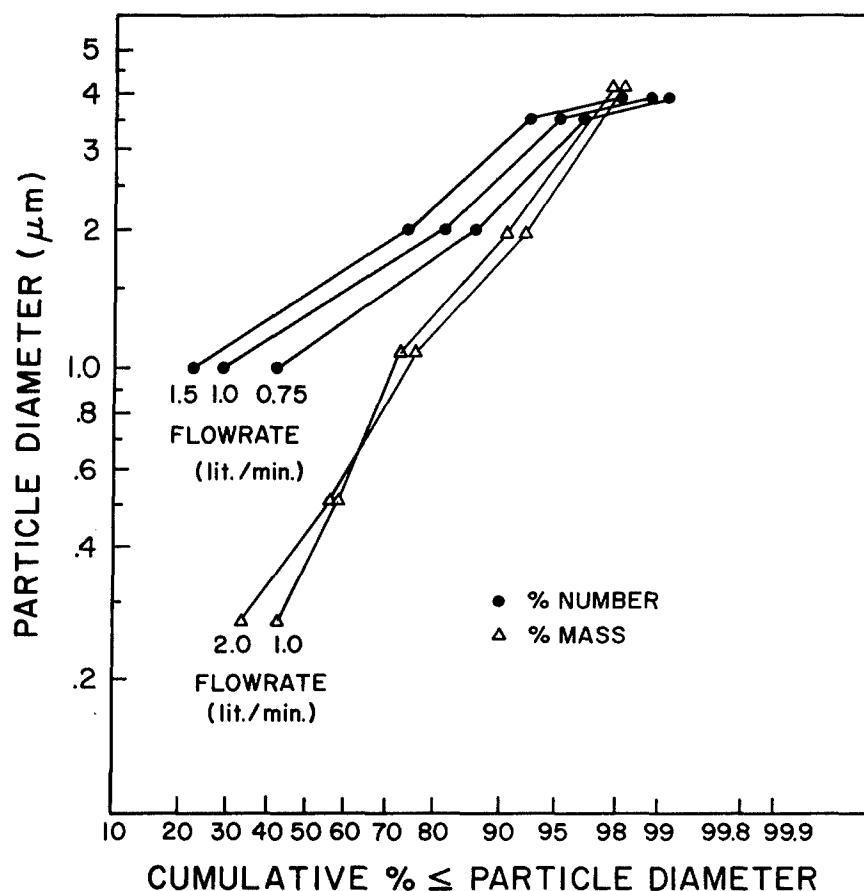


Figure 10.
Particle generator calibration:
size distribution
by % mass and %
number at differ-
ent delivery rates.
Mean diameter =
50%. % mass = 2
reps per point.
% number = 3 reps
per point.

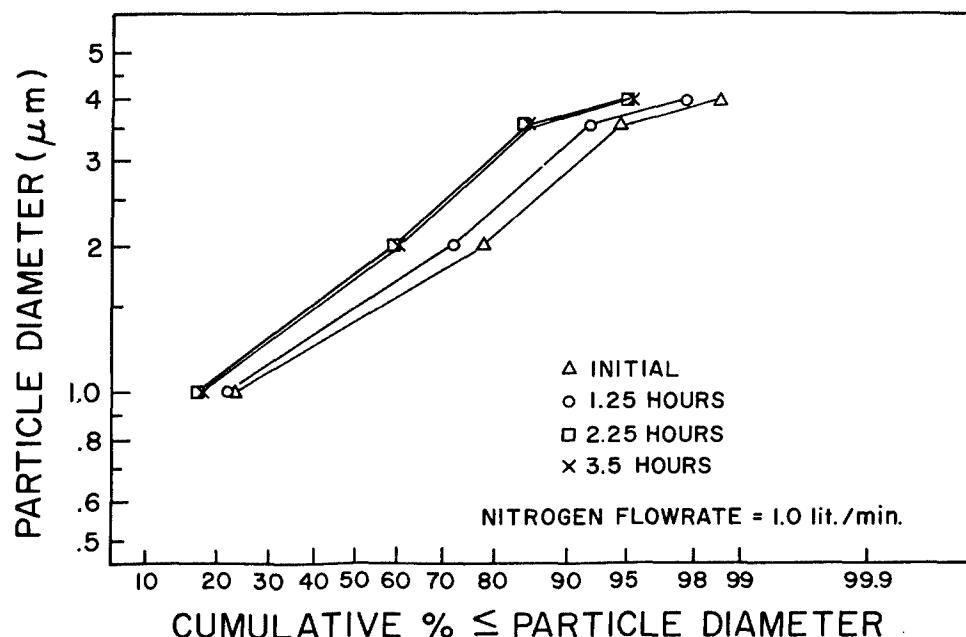


Figure 11.
Particle gen-
erator calibra-
tion: size dis-
tribution by %
number at four
points during
3.5 hours of
continuous
operation. 3
replications
per point.

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RESPONSE OF SEVERAL PLANT SPECIES TO SHORT EXPOSURES
OF HYDROGEN CHLORIDE GAS

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INTRODUCTION

Hydrogen chloride (HCl) gas is a major exhaust product of solid fuel rockets (Final Environmental Statement USAF Space Launch Vehicles, 1975; Gregory and Storey, 1975; Wagner, 1975). During launch tremendous amounts of the gas are released; most follow the rocket to the upper atmosphere where they are dispersed by the winds. The remainder, depending on current weather conditions, can become entrapped in a cloud that may remain intact for several minutes to several hours. Before being dispersed by ground winds, the cloud may touch down on land. What happens when a high concentration cloud briefly passes over or onto vegetation is of concern to us.

We are assuming several factors in our investigations: that the cloud will remain on any one plant 30 minutes or less; that the concentration of HCl will remain reasonably constant during that time; and that HCl is the major phytotoxic component of the cloud. The validity of these hypotheses is being tested by us or others. The present studies are short exposures of certain plants to nearly constant, known concentrations of HCl gas. Attempts are made to limit exposures to periods when the temperature, relative humidity, and light intensity are within certain values. The plants under test are those which may be found commercially or otherwise in the Lompoc Valley east of Vandenberg AFB. This area is famous for its flower

seed production. Our first interests have been to detect obvious visible injury on the plants exposed and to determine the times and concentrations necessary for such injury. There were early indications that the nutrition a plant receives affects that plant's tolerance to phytotoxic gases. The relationship between nutrition and tolerance is briefly reported here.

MATERIALS AND METHODS

PLANTS

Flower and vegetable plants were grown from seed in germinating soil and transplanted to 10 cm pots of UC mix (Lerman, 1975). The plants included barley, bean, centaurea, marigold, nasturtium, radish, tomato, and zinnia. Plants were grown in greenhouses equipped with activated charcoal filters and evaporative coolers. During summer months the greenhouses were whitewashed to prevent excessive temperatures. The plants were grown for different periods depending on the experiment.

EXPOSURE EQUIPMENT

Plants were placed in 0.45 m^3 chambers covered with mylar sheeting. HCl gas was generated by volatilizing aqueous acid solutions into a constant air flow as previously reported (Granett and Taylor, 1976; Mandl et al., 1971). Special plant racks and a counter-balanced door allowed rapid insertion and removal of plants. Light intensity, temperature, and relative humidity were recorded for each exposure. During exposure 15 l of chamber air was drawn through 20 ml of weak acid (0.1 N HNO_3) and this solution was later analyzed for chloride ion content with an American Instruments Automatic Chloride Titrator (Model 4-4433).

EXPERIMENTAL PROCEDURES FOR NUTRIENT HYDROPONIC EXPERIMENTS

In this experiment nasturtium plants were grown hydroponically with nutrients circulating around the plant roots (Thomas, 1976)(see Figure 1). The plants were supported in wood and cork discs which in turn fitted into large tee-fittings in 10 cm PVC

tubing. Nutrients were pumped through the tubing from a reservoir. Thirty-four-day-old plants were removed to empty 10 cm pots for fumigations. Since visible injury occurred on plants supplied with normal nutrients at HCl concentrations of 20-22 mg/m³, 20-minute exposures at this concentration was the dosage used for all tests. The nutritional makeup of the hydroponic solution was varied for different studies and the degree of injury was measured.

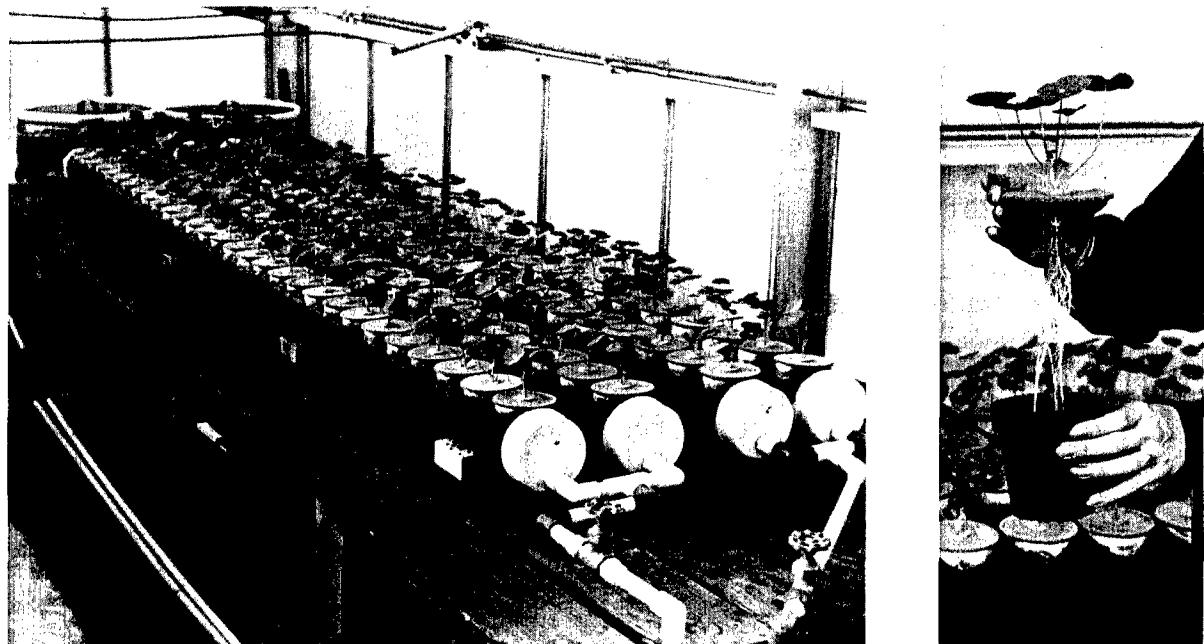


Figure 1. Left: Hydroponic system of 10 cm PVC pipe for growing nasturtium plants in nutrient solutions. Right: Plant is supported in a 10 cm pot during fumigation.

EXPERIMENTAL PROCEDURES FOR NUTRITION LEVEL EXPERIMENTS

In another series of experiments, treatments consisted of one of three volumes of the balanced nutrient solution being applied to the plants each week. The plants, which were grown in sterile sand, included marigolds and nasturtium. Fumigations consisted of 20-minute exposures to one of several concentrations

of HCl gas. Plants were measured just before fumigation, then harvested 2-3 days postexposure when visible injury had fully developed.

EXPERIMENTAL PROCEDURES FOR PLANT SENSITIVITY EXPERIMENTS

In the sensitivity tests, plants were grown to a standard age at which time they were fumigated once for 5 to 30 minutes. The HCl gas concentration of successive exposures was adjusted to approach a threshold level at which plants were just beginning to show visible damage; higher levels produced severe leaf damage, while lower HCl levels produced almost no damage. Damage was rated either on the basis of all 12 plants in a fumigation (injury or no-injury per exposure) or on the number of leaves injured. If enough plants were tested, the data could be computer analyzed to yield lines of no injury and 1-leaf injury. The area between the two curves represented threshold dosages.

PLANT VARIABILITY AND EXPERIMENTAL CONTROLS

Before discussing the individual tests, the variability among plants should be mentioned. Plants may react to fumigants differently, in response to light, temperature, and/or humidity. The natural variability of the plant, however, must also be taken into account. For example, bean plants exposed at the same time showed considerable variation in area with necrotic damage. Other leaf damage such as glazing, flecking, chlorosis, and discoloration would also vary from leaf to leaf when it occurred. For these reasons, we have been trying to avoid estimating total damage, relying instead on number of leaves injured or number of plants injured. This method has the advantage of accurate interpretation by any other laboratory. In the hydroponic experiments, however, damage index (DI) was determined by the formula $DI = 2N + G + D$, where N is percent leaf area with necrosis, G is percent leaf area with abaxial glazing, and D is percent leaf area with leaf discoloration (Thomas, 1976).

During the course of these experiments various controls have been used. Some plants were not fumigated, that is, they were left on the greenhouse bench. Other control plants were placed in the fumigation chamber and exposed to 0 mg/m³ HCl gas for the required time. Several hundred plants thus far exposed

have no visible damage. It is doubtful if the chamber air passing over the plants has any long-term growth effect, although this has not been rigorously tested.

RESULTS

NUTRIENT HYDROPONICS

When relative calcium, magnesium, potassium, and chlorine concentrations were altered in the nutrient solution, exposed plants responded differently (Thomas, 1976). The damage is summarized in Table 1. The elements acted differently. Calcium-deficient plants had some tolerance to damage, whereas plants supplied with excessive amounts of the same element became more sensitive to injury. Damage indexes for either calcium treatment were significantly different from the control indexes. Plants were more sensitive to damage with both excessive and deficient amounts of chlorine in the nutrient solution. There was increased susceptibility to damage as the chlorine level increased above normal levels. Magnesium also brought about increased sensitivity at both extremes but this sensitivity was most pronounced (and damage was significantly greater than the control) in the plants deficient in magnesium. None of the potassium-treated plants differed significantly from the controls although plants receiving either extreme treatment were damaged more than the control plants.

TABLE 1. DAMAGE INDUCED ON NASTURTIUM PLANTS EXPOSED TO HCl GAS FOR 20 MINUTES AFTER GROWING HYDROPONICALLY IN DIFFERENT NUTRIENT SOLUTIONS

	<u>Relative Strength of Element in Nutrient</u>		<u>HCl Gas Conc.</u> (mg/m ³)	<u>(ppm)</u>	<u>Damage Index</u>
Calcium	½	(control)	21.0	(13.8)	3.09**
	1		21.0	(13.8)	32.12
	2		21.0	(13.8)	62.72**
Chlorine	0	(control)	22.0	(14.7)	14.7**
	1		22.0	(14.7)	5.5
	2		22.0	(14.7)	8.1
	3		22.0	(14.7)	10.5
	4		22.0	(14.7)	10.1
	5		22.0	(14.7)	17.6**
Magnesium	½	(control)	22.5	(14.8)	3.25**
	1		22.5	(14.8)	1.03
	4		22.5	(14.8)	1.52
Potassium	½	(control)	20.8	(13.7)	0.98
	1		20.8	(13.7)	0.84
	4		20.8	(13.7)	1.82

**Significantly different from control of same group at 1% level by Dunnett's all vs. control test.

NUTRITION LEVEL

In addition to the above study, the effect of the total nutrient level on plant sensitivity was investigated. Marigold and nasturtium plants were grown in sand, and water was supplemented with three levels of Hoagland's nutrient solution (Hoagland and Arnon, 1938) totaling 40, 80, and 160 ml per pot per week. The normal nutrient supplementation is 80 ml per week. Table 2 summarizes the growth of the plants in the three nutrient treatment groups. For nasturtium there was excellent linear correlation when either leaf numbers ($r=0.91$) or fresh weight of plant leaves and stems ($r=0.87$) were compared to nutrient treatment. For the marigolds, the weight was a better measure of the effect of the nutrient on the plant growth ($r=1.00$). Leaf number did not correlate well ($r=0.14$) nor did plant height. The dry weights of the stems and leaves of each plant group also correlated well with nutrient treatment.

TABLE 2. EFFECT OF NUTRIENT TREATMENT ON PLANT GROWTH

<u>Plant</u>	<u>Growth Factor</u>	<u>Nutrient Treatment¹</u>			<u>Correlation Coefficient (r)</u>
		<u>40</u>	<u>80</u>	<u>160</u>	
Nasturtium	Leaves ²	27.3	35.4	38.8	0.91
	Weight ³	5.9	9.8	10.8	0.87
Marigold	Leaves ⁴	15.4	16.0	15.6	0.14
	Weight ⁵	11.6	13.5	16.6	1.00

¹ml Hoagland's nutrient solution per plant per week.

²Mean number of leaves per plant for 60 plants.

³Mean fresh weight in grams of stems and leaves from 60 plants.

⁴Mean number of leaves per plant for 75 plants.

⁵Mean fresh weight in grams of stems and leaves from 25 plants.

When the plants were about 6 weeks old, they were exposed to 20 minutes of one of several different concentrations of HCl gas. After 24 hours the plants were graded for damage. The most useful damage index was the percent of the total leaves showing any injury. Figures 2 and 3 and Tables 2, 3, and 4 summarize the damage data. Marigolds were damaged more severely

than nasturtiums. The damage correlated well with both treatments. There is evidence here that visible leaf damage caused by exposure to HCl gas is related to the amount of nutrient the plant has received. Sensitivity to injury is apparently increased when plants are already stressed with sub-optimal amounts of nutrients.

TABLE 3. DAMAGE ON PLANTS GROWN UNDER DIFFERENT NUTRIENT CONDITIONS AND THEN EXPOSED TO HCl GAS

<u>HCl Gas Concentration¹</u> (mg/m ³)	<u>Concentration¹</u> (ppm)	<u>Nutrient Treatment²</u>		
		<u>40</u>	<u>80</u>	<u>160</u>
NASTURTIUM				
0	(0)	0 ^{3, 4}	0	0
4.4	(2.9)	0	0	2
14.5	(9.5)	2	0	1
25.7	(16.9)	30	14	9
MARIGOLD				
0	(0)	0 ⁵	0	0
7.4	(4.9)	7	6	0
14.4	(9.5)	25	23	11
22.1	(14.5)	41	29	19
31.0	(20.4)	67	67	52

¹Each exposure lasted 20 minutes.

²ml Hoagland's nutrient solution per plant per week.

³Percent leaves with any damage.

⁴Mean of 25 plants.

⁵Mean of 15 plants.

TABLE 4. CORRELATION COEFFICIENTS (r) OF NUTRIENT AND FUMIGATION TREATMENTS OF NASTURTIUM AND MARIGOLD PLANTS

	<u>Treatment</u>	
	<u>Nutrient</u>	<u>HCl Concentration</u>
Nasturtium	-0.86	0.87
Marigold	-1.00	0.99

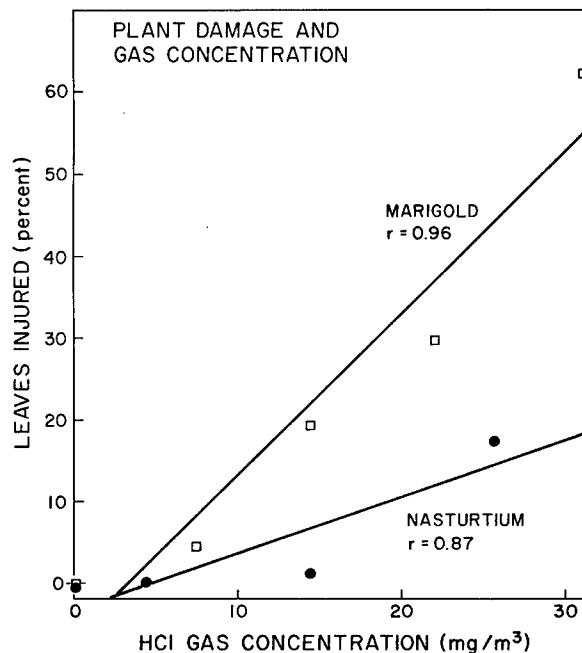


Figure 2. Regression lines showing strong positive correlation between the gas concentration to which marigold (□) or nasturtium (●) plants are exposed for 20 minutes and the percent of total leaves that are injured. ($\text{ppm} = 0.658 \text{ mg}/\text{m}^3$)

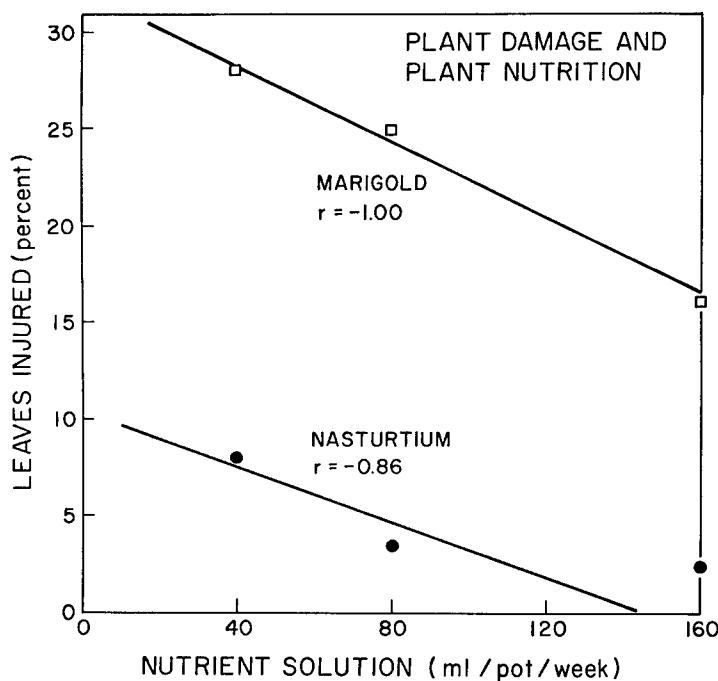


Figure 3. Regression lines showing a strong negative correlation between the total amount of a complete nutrient solution that nasturtium and marigold plants receive and the amount of injury the plants sustain when exposed to HCl gas at phytotoxic levels for 20 minutes.

PLANT SENSITIVITY

Table 5 summarizes the data accumulated on the sensitivity of six species. These concentrations were derived from single exposures of many plants to different concentrations of HCl. For barley and bean, over 500 plants each were used. The data for those two plants were analyzed with a computer program which fitted the data to a hyperbolic regression. The equations derived are presented in Table 6.

TABLE 5. SENSITIVITY OF PLANTS EXPOSED TO
SHORT DURATION OF HCl GAS

<u>Species</u>	Exposure Time (min)					<u>20</u>		
	<u>5</u>	<u>10</u>	<u>15</u>	<u>20</u>				
Barley	28	(18) ¹	16	(11)	13	(9)	10	(7)
Bean	21	(14)	14	(9)	12	(8)	11	(7)
Centaurea	25	(16)	25	(16)	<30	(<20)	<23	(<15)
Nasturtium	>50	(>33)	30	(20)	<48	(<32)	30	(20)
Radish	22	(14)	16	(11)	12	(8)	10	(7)
Tomato	>23	(>15)	>22	(>14)	>19	(>12)	<15	(<10)

¹Estimates of minimal concentration of HCl gas in mg/m³ (ppm) necessary for visible leaf injury. Symbols (>, <) refer to insufficient data above or below the level listed.

TABLE 6. COMPUTER DERIVED SENSITIVITY CURVES FOR
BARLEY AND BEAN PLANTS EXPOSED TO HCl GAS

	<u>Barley</u>	<u>Bean</u>
Equation ¹	$I = 0.415 - 0.085(c) - \frac{9.588}{T}$	$I = -0.487 + 0.070(c) - \frac{5.003}{T}$
No. Plants Fumigated	672	504
Correlation Coefficient (r)	0.52	0.66

¹Where I = injury, c = concentration HCl in mg/m³, and T = exposure time in minutes.

Considering the number of plants used, there is good correlation with the curves. Figure 4 shows how the sensitivity curves appear when plotted over the fumigation data. The data points in Figures 4 and 5 refer to whether any of the 12 fumigated plants were damaged (+) or not (0). The curves, Figure 4, are computed for one-leaf (+1) or no-leaf (+0) damage. Figure 5 represents the data for the four other species, centaurea, nasturtium, radish, and tomato.

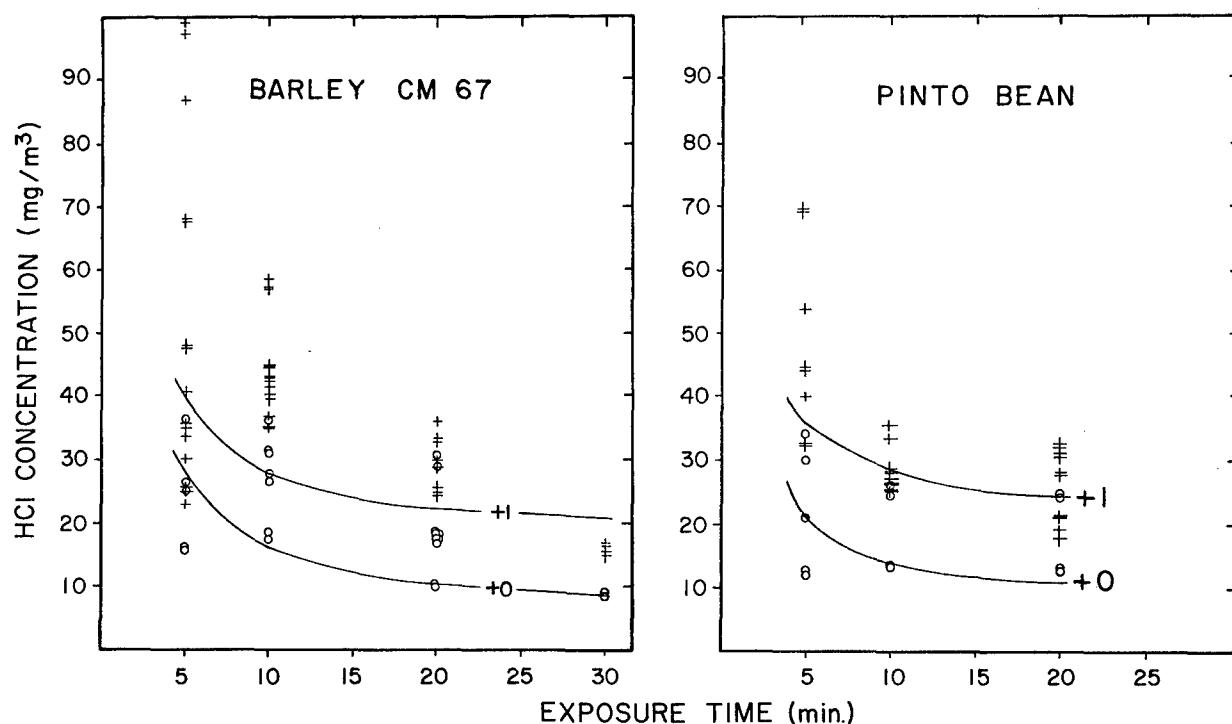


Figure 4. The sensitivity of barley and bean plants to short durations of HCl gas. Each point represents 12 plants. + = damage on 1 to 12 plants, 0 = no damage. Curves are calculated for one leaf (+1) or no-leaf (+0) injury per plant.
(ppm = 0.658 mg/m³)

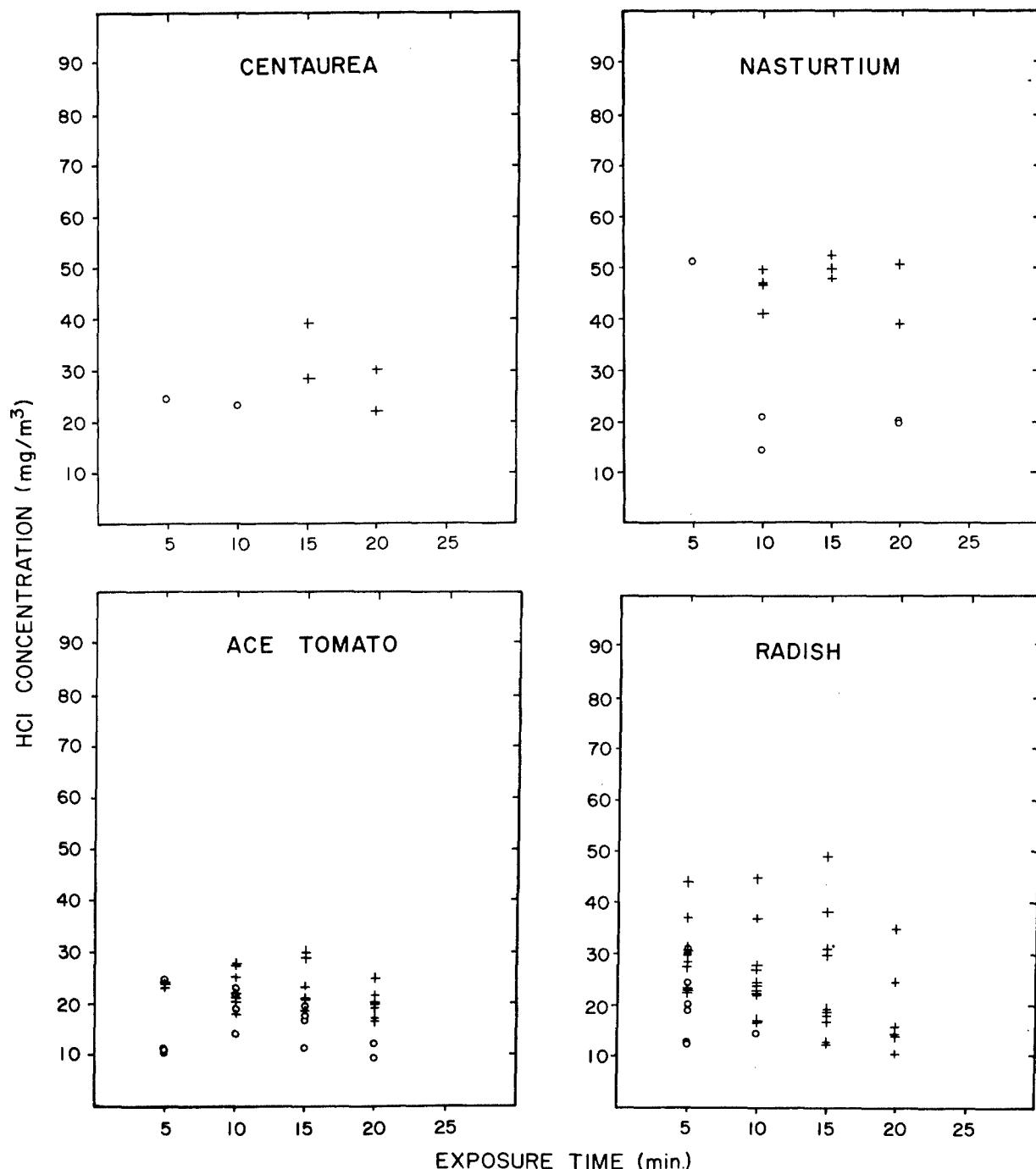


Figure 5. The sensitivity of four species to short durations of HCl gas. Each point represents 12 plants. + = damage on 1 to 12 plants, o = no damage. (ppm = 0.658 mg/m³)

The leaf damage data for radishes is also presented in Table 7. The number of plants in each dosage (time-concentration combination) was 60 to 325.

TABLE 7. LEAF DAMAGE ON RADISHES EXPOSED TO HCl GAS

<u>HCl Conc.¹</u>	Exposure Time (min)			
	<u>5</u>	<u>10</u>	<u>15</u>	<u>20</u>
11-20 (7-13)	--	--	48 ²	27
21-30 (14-20)	4	26	--	--
31-40 (21-26)	7	58	70	61
41-50 (27-33)	16	38	85	--

¹Gas concentration in mg/m³ (ppm).

²Percent total leaves with any damage.

Zinnia seedlings were fumigated at 5 different ages at comparable time-concentration combinations. The age of the plants ranged from 8 to 41 days and there was good linear correlation ($r=0.87$) of age to number of leaves (Table 8). The oldest plants, however, were large and presented problems. They took up much space in the fumigation chamber and their large surface area affected the fumigant concentration. The 27-day-old plants had single, unopened flower buds and the 41-day-old plants had open flowers and a number of axillaries with flowers. Table 9 summarizes the data for the damage at different ages. Damage of the leaves correlated very well ($r=0.98$) with concentration (Figure 6). There is also a fair correlation ($r=0.88$) of damage with age, with a strong indication that older plants are less sensitive than younger plants (Figure 7). Plants 22 days old seemed most sensitive as they sustained the most damage.

TABLE 8. NUMBER OF LEAVES ON ZINNIA PLANTS OF DIFFERENT AGES

	Age (days)				
	<u>8</u>	<u>16</u>	<u>22</u>	<u>27</u>	<u>41</u>
Leaves per plant	4	10	12	14	14
Leaves fumigated	56	140	168	192	196

TABLE 9. DAMAGE ON ZINNIA PLANTS EXPOSED TO HCl GAS AT DIFFERENT AGES

Range	Concentration ¹ Mean	Age (days)					Average Damage Per Concentration Level ⁴
		8	16	22	27	41	
0	0 (0)	0 ²	0	0	0	0	0
1-10	9.3 (6.1)	9.0	--	--	3.3	4.3	5.5
11-20	14.2 (9.3)	--	2.7	1.3	15.3	8.3	6.9
21-30	23.2 (15.3)	45.0	19.3	20.0	--	25.3	26.9
21-40	37.0 (24.3)	--	--	48.7	45.7	--	47.2
41-50	44.5 (29.3)	82.0	46.3	--	--	--	64.2

Average damage per exposure time ³	34.0	17.1	21.2	16.0	9.4
---	------	------	------	------	-----

$r=0.98$

$r=0.88$

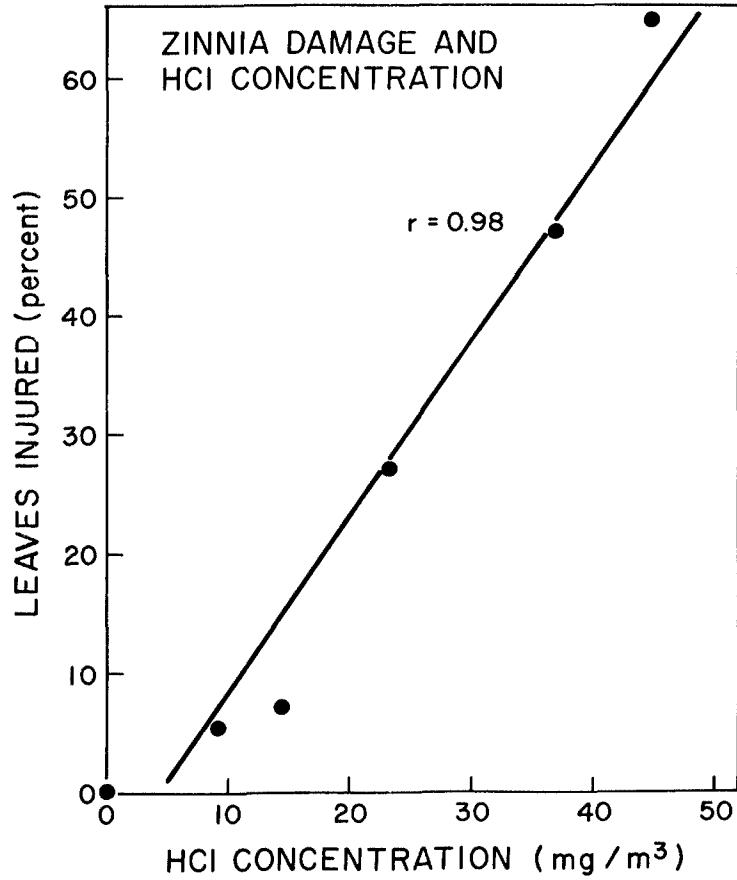
¹HCl concentration in mg/m³ (ppm).²Damage is percent of total leaves injured.³Mean of exposure damages.⁴Mean of concentration damages.

Figure 6. Regression line showing strong positive correlation between HCl gas concentrations to which zinnia plants are exposed and the percent of total leaves which are injured. HCl concentration is the mean of the concentrations for different exposure durations. (ppm = 0.658 mg/m³)

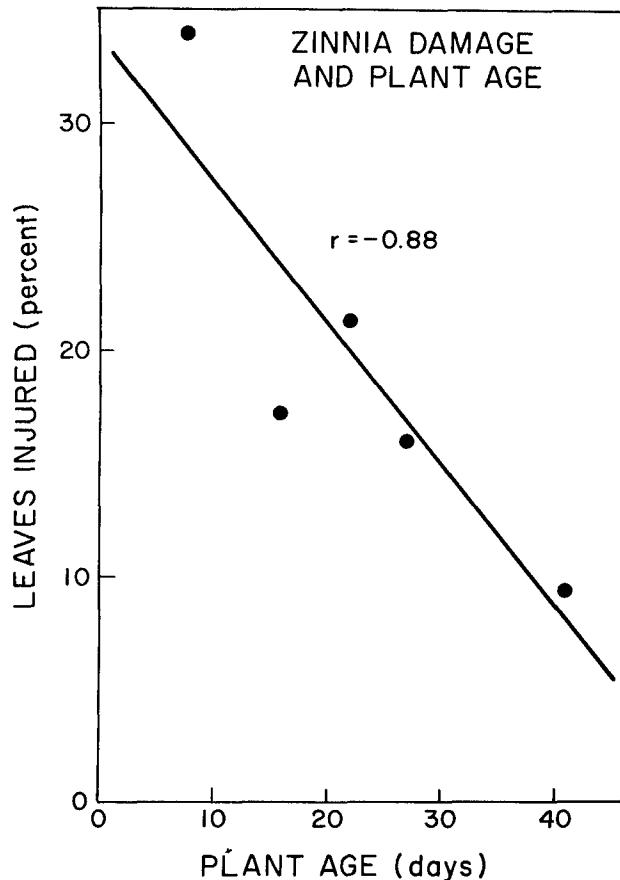


Figure 7. Regression line showing good negative correlation between the age at which zinnia plants were exposed to HCl gas and the percent of total leaves which were injured. The HCl concentration and duration of exposure were averaged.

In Table 10, the damage is summarized for different exposure durations. Here there is excellent correlation between length of exposure and damage (Figure 8).

TABLE 10. DAMAGE RESPONSE OF ZINNIA PLANTS TO
SHORT DURATION EXPOSURES OF HCl GAS

Concentration ¹ Range	Mean	Exposure Time (min)			Average Damage Per Concentration Level ⁴
		5	10	20	
0	0 (0)	0 ²	0	0	0
1-10	9.3 (6.1)	0	2.8	14.0	5.6
11-20	14.2 (9.3)	1.0	2.8	17.0	6.9
21-30	23.2 (15.3)	5.0	23.8	52.0	26.9
31-40	37.0 (24.3)	17.5	47.5	76.5	47.2
41-50	44.5 (29.3)	43.5	69.0	80.0	64.2

Average damage per exposure time ³	11.2	24.3	39.9	r=0.98
				r=0.99

¹HCl concentration in mg/m³ (ppm).

²Damage is percent of total leaves injured.

³Mean of exposure damages.

⁴Mean of concentration damages.

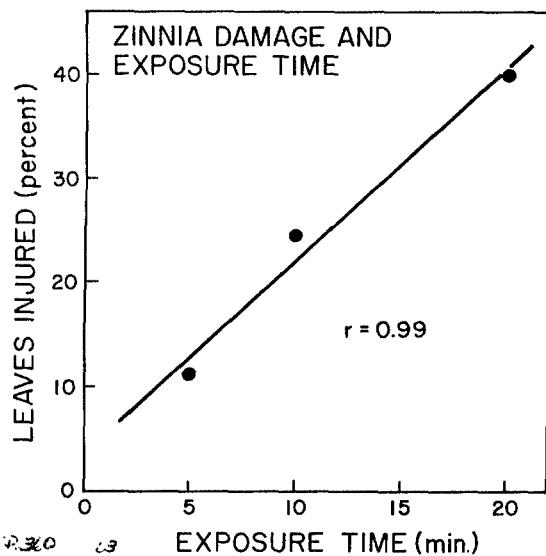


Figure 8. Regression line showing strong positive correlation between the length of time zinnia plants are exposed to HCl gas and the percent of total leaves which are injured. HCl concentrations were averaged for each exposure time.

DISCUSSION

From these studies it is apparent that there is a relation between the nutrition supplied to a plant and its tolerance or sensitivity to HCl gas. Predictably, the total nutrition level affected the plant's growth. In addition, less HCl-induced damage was seen on the nasturtium and marigolds favored with more of the complete nutrient solution. In contrast, in the hydroponics experiments, in which the concentrations of specific elements supplied to nasturtiums were varied, there was greater sensitivity to damage. In particular, chlorine- or magnesium-deficient plants and those receiving chlorine and calcium in excessive amounts were more sensitive to gas-induced injury. Calcium-deficient nasturtiums were the only plants which showed increased tolerance to HCl damage. Excessive magnesium did not seem to be significant. The complete nutrition treatments may have been less than optimal, even at the high (160 ml/wk) level. Leone (1976, 1970) used 300 ml of full strength nutrient solution every other day for tomato seedlings. Potassium had no significant influence on resistance in nasturtium. Leone (1976), on the other hand, has found significant reduction in visible injury in potassium-deficient, ozone-treated plants but she used more extreme treatments than we did.

The sensitivity of a plant to HCl gas has been explored in several ways in these experiments. The computer-derived curves are impressive but they require a large number of plants to produce. It is doubtful whether they provide more useful conclusions than when the gas concentrations are grouped in discrete ranges and the damage levels recorded in a table such as was done for the radish and zinnia data.

The zinnia work included data on the sensitivity of plants at different ages. The younger, vegetative stages appeared most sensitive with a decrease in sensitivity as the leaves become more mature. On older plants the more sensitive leaves were those on axillary shoots.

From our evidence, injury does not seem to occur on any plants at HCl concentrations below about 10 mg/m³. This is well above gas concentrations estimated to be present at sites near launch areas (Gregory and Storey, 1975; Wagner, 1975). Although increased fertilization may decrease damage from HCl gas, this would probably not be economically feasible for most crops.

This work provides the basis for further tests with HCl gas. The sensitivities of more species must be tested as well as the range of tolerance found in varieties of the same species. There is evidence that plants grown outside the filtered-air greenhouse are more resistant to HCl gas damage and this effect on a plant's sensitivity must be studied. The influence of climatic factors and the long-range effects of injury induced by HCl gas must also be considered.

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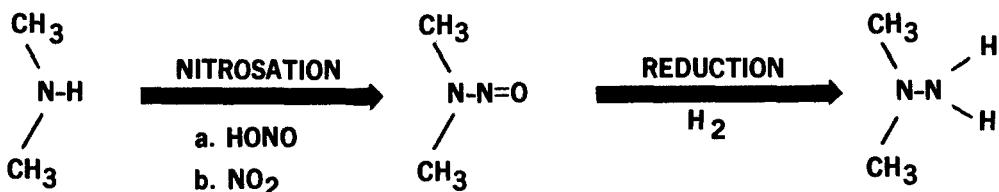
COMPARISON OF ANALYTICAL METHODS FOR MEASUREMENT OF
 N-NITROSODIMETHYLAMINE IN
 1,1-DIMETHYLHYDRAZINE-ENRICHED ATMOSPHERES

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INTRODUCTION

Fine et al., (1976a, 1976b) and Pellizzari et al., (1976b) have recently reported on the detection of N-nitrosodimethylamine (NDMA) in ambient air using extremely sensitive techniques. N-nitrosodimethylamine is one of fourteen chemical carcinogens regulated by the Occupational Safety and Health Administration and is an intermediate in the industrial synthesis of 1,1-dimethylhydrazine (UDMH) rocket fuel (Figure 1).



	DMA	NDMA(DMN)	UDMH
MW	45.09	74.08	60.11
bp°C	7.4	154	63
Sp. gr.	0.68	1.01	0.78

Figure 1. Industrial synthesis of UDMH.

By interagency agreement, the Air Force is the primary supplier of propellant UDMH for all national defense and space applications. Because of the potential impact of the recent measurements on continued UDMH production, the Aerospace Medical Division in late 1975 undertook an evaluation of new NDMA measurement techniques, for possible augmentation of ongoing environmental sampling programs. This paper presents results of a low level NDMA study conducted to compare three different sampling and analytical procedures under controlled laboratory conditions.

The evaluation included (a) 3-stage cryogenic sampling with analysis by coupled gas chromatography-mass spectrometry (GC-MS); (b) NDMA concentration on Tenax-GC cartridges with analysis by GC-MS after the method of Pellizzari et al., (1976a), and (c) impinger sampling with analysis by the Thermal Energy Analyzer (TEA) after the method of Fine and Rounbehler (1975b). The laboratory facility chosen for this comparison was a toxic hazards exposure chamber (Thomas Dome) at the Aerospace Medical Research Laboratory, Wright-Patterson Air Force Base, Ohio. This facility was selected for two reasons. First, it afforded a reliable and safe method for generating minute concentrations of NDMA in chamber air. Secondly, the experimental protocol provided a check on chamber atmospheres employed in several UDMH animal exposure experiments conducted at Wright-Patterson in 1975.

The atmosphere selected for the comparison study was UDMH-in-air at a concentration of 5 parts per million (ppm) by volume. This concentration was the highest level of UDMH employed in the 1975 animal experiments, and corresponded to ten times the current Threshold Limit Value established by the American Conference of Governmental Industrial Hygienists. The UDMH material used for the comparison test was of two compositions. The first was "pure" UDMH obtained from distillation of a mixed amine fuel (MAF-1) reclaimed from the Bullpup missile program. The pure UDMH had been previously analyzed by mass spectrometry and found to be free of NDMA contamination. The second UDMH composition was the same Bullpup UDMH artificially enriched to 1200 mg/liter (0.12% v/v) in NDMA. This composition was similar to a production lot UDMH that had been employed in several of the initial UDMH animal exposure experiments (Haun, 1976).

METHODS AND MATERIALS

EXPERIMENTAL SETUP

The experimental setup in the toxic hazards exposure chamber is shown schematically in Figure 2. Vapor phase UDMH was added to the chamber by syringe injection of liquid UDMH into a continuous stream of air flowing at a rate of 1.7 cu m/minute (60 CFM). The chamber volume was 23.8 cu m (840 cu ft) which gave a nominal air residence time of 14 min. The chamber itself was clean and empty throughout the test. Sampling lines for each of the three collection systems were made through separate penetrations in the chamber wall, and tied inside to a common inlet point (approximately 1 meter high and 1 meter from the wall). The sample tubing was 6.4 mm O.D. polyethylene (Imperial Eastman Poly-Flow). The sample flow rate was 0.3 liters per minute for the cryotrap; 1.0 liters per minute for the Tenax-GC cartridges, and 5.0 liters per minute for the bubblers.

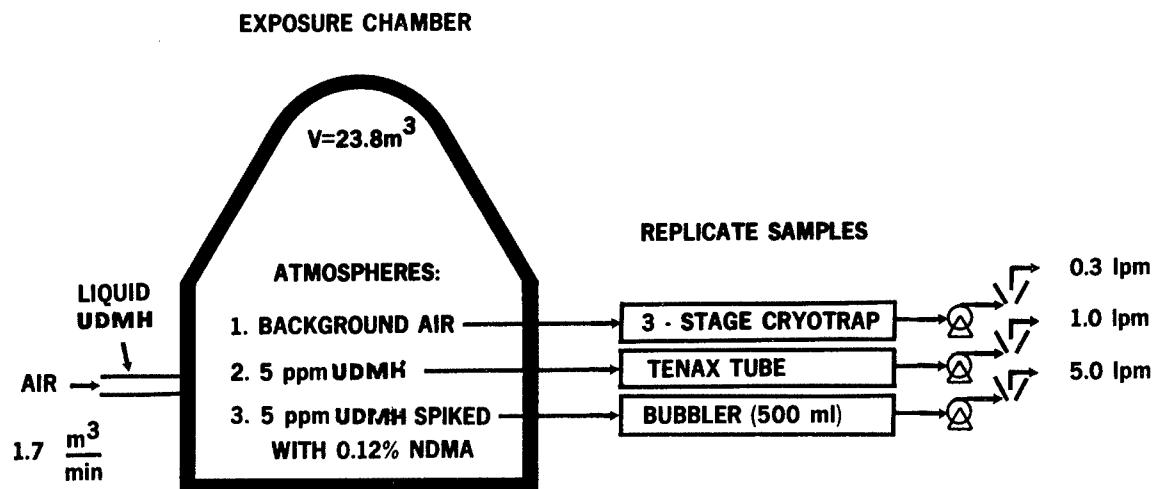


Figure 2. Schematic of experimental sampling setup in toxic hazards exposure chamber.

DESCRIPTION OF SAMPLING APPARATUS

Cryotrap

The USAFSAM 3-stage cryogenic sampling system has been described by Conkle et al. (1974) and is shown schematically in Figure 3. Briefly, the system employs three cold traps in series. The first is maintained at 0°C (ice water), the second at -78°C (pulverized dry ice), and the third at -175°C (liquid nitrogen). Two unique features of the cryosystem are a heated inlet to the dry ice trap to prevent ice blockage, and an annular gaseous nitrogen flush in the LN₂ trap to prevent condensation of liquid oxygen. The sample cylinders are nominal 150 cc volume made of 316 stainless steel. Atmospheric sample was drawn from the chamber through the traps by a metal bellows pump, and exhausted through a flowmeter. Flow control was obtained by needle valve located between the pump and flowmeter. Cryotrap pressure was monitored for sample volume correction by an absolute pressure gauge (Wallace & Tiernan Model FA-160) mounted on the pump inlet.

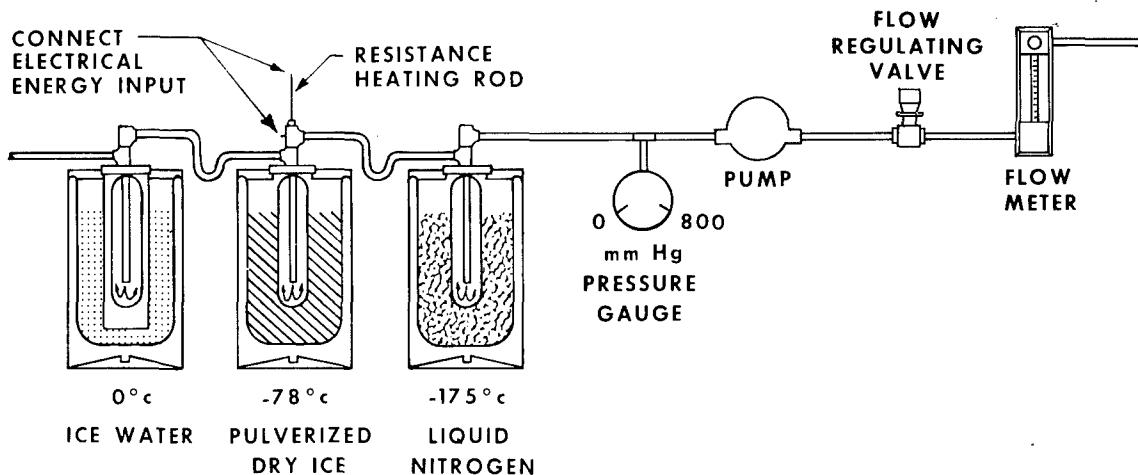


Figure 3. Schematic flow diagram for 3-stage sampling system.

Thorough cleaning of the trap cylinders has been found to be essential for accurate trace analytical work. Each cylinder was flushed for 8 hours with 150°C superheated steam, filled to 50 psig with helium and checked for zero leakage. The cylinder

was then placed on a manifold and evacuated to 10^{-6} Torr under a heating blanket at 100 C. Finally, the cylinder was flushed with zero grade helium, filled to 50 psig and the contents checked gas chromatographically. Each cylinder was transported to the field under helium.

Tenax-GC Cartridges

The use of Tenax-GC (2,6-diphenyl-p-phenylene oxide) polymer sorbent sampling cartridges for NDMA detection has been described by Pellizzari et al. (1976a). The Tenax sampling cartridges employed for our study were similar but differed in two respects. Whereas their cartridges were glass tubes with glass wool retainer plugs, ours were 316 stainless steel tubes with stainless steel screen retainers. The use of stainless steel tubes with permanent couplings prevented transport breakage and reduced the opportunity for sample contamination. Similarly, the modification to stainless steel screen retainers was made when glass wool was found to be an occasionally effective but erratic sorbent for organic compounds.

Our Tenax cartridges (Figure 4) were 15.2 cm (6 inch) lengths of 12.7 mm O.D. ($\frac{1}{2}$ -inch) stainless tubing. The interior wall was electropolished to reduce surface effects. Each end of the tube was fitted with a cap (Swagelok SS-810-C) which had been modified by addition of a welded vacuum coupling (Swagelok 4V-CR-3-A-SS) and vacuum nut (Swagelok 4V-CR-1-SS) to accept a removable Teflon washer and nylon plug. This configuration permitted ready connection to either the sampling pump or desorption manifold. Each cartridge contained approximately 2.5 grams of Tenax-GC (35/60 mesh) purchased from Applied Science Laboratories, Inc., State College, Pennsylvania 16801. Prior to use each batch of Tenax was baked at 150 C in a vacuum oven for 24 hours. The cartridge was then loaded, placed in a heating manifold (Figure 5) and thermally desorbed at 240 C with a 30 cc/min helium flush until no trace peaks appeared on gas chromatographic check. The cartridge was then capped and transported to the field under helium blanket.

The Tenax collection system consisted of cartridge, sampling pump and flowmeter, in that order. A ball valve and filter (7-micrometer) were located between the cartridge and pump to check for leaks and to prevent pump contamination by dust, respectively. Sample flow was regulated at 1.0 liters per minute by needle valve between the pump and flowmeter.

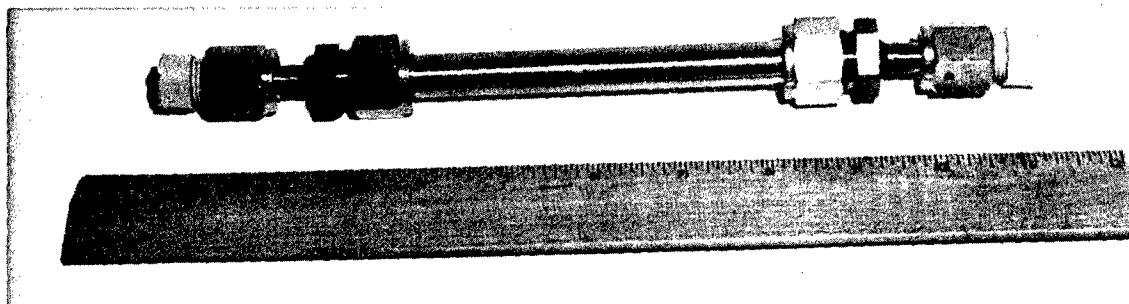


Figure 4. Tenax-GC sampling cartridge.

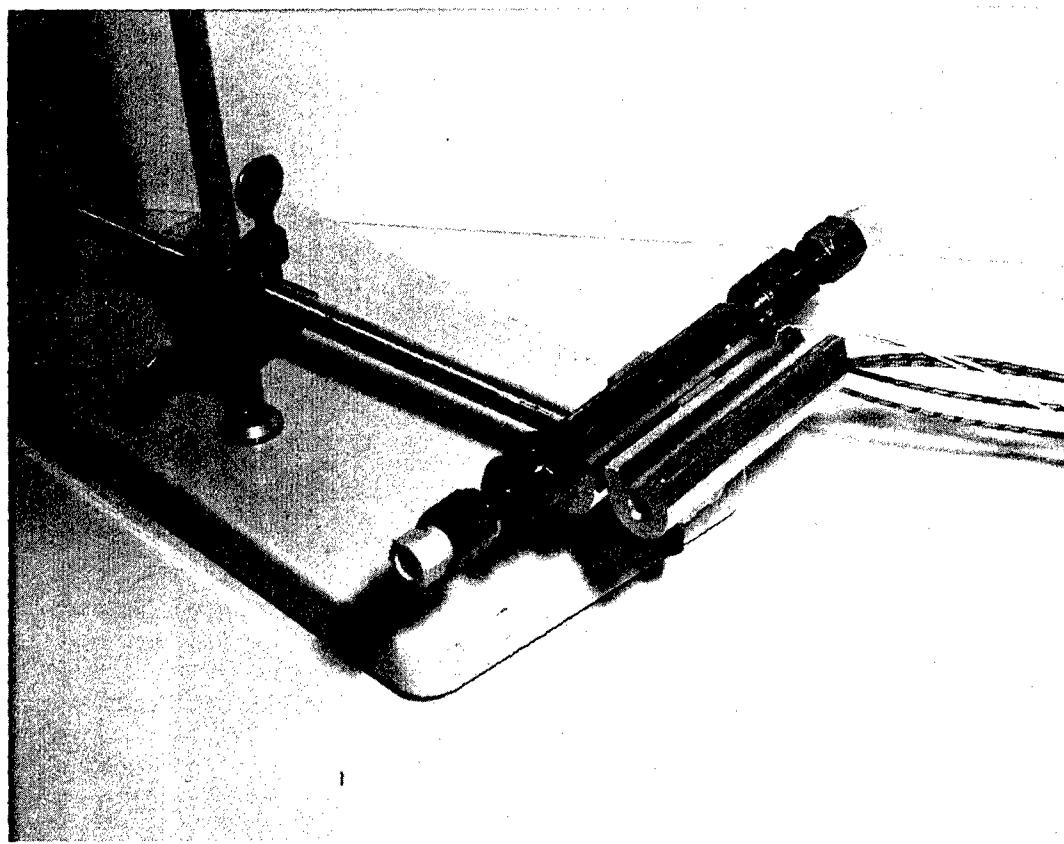


Figure 5. Manifold for thermal desorption of Tenax-GC sampling cartridge.

Impinger Sampling

Impinger sampling was done for NDMA analysis by coupled gas chromatography-thermal energy analyzer (GC-TEA). The impingers were standard Greenberg-Smith configuration, 500 ml capacity, and contained 100 ml of 1 N potassium hydroxide. Two impingers, in series, were used for each sample. The impingers were connected to a downstream ball valve, sampling pump, needle valve and flowmeter, in that order. The sample flow rate was 5.0 liters per minute for one hour and was regulated by needle valve. At the completion of the sampling period the 1 N KOH solution in the two impingers was combined, transferred to a polyethylene bottle and frozen until analysis.

ANALYTICAL PROCEDURES

GC-MS-Data System

Analysis of the Tenax-GC and cryogenically collected air samples was accomplished with a coupled gas chromatograph-mass spectrometer-data system (DuPont Model 21-491). The separation packing was Porapak Q (100/120 mesh) in a 3 m long by 1.6 mm O.D. Microbore stainless steel column. The carrier gas was helium at a flow rate of 30 cc/min, and the chromatograph was temperature programmed from -100 C to 240 C at 10 C/min. The chromatographic effluent was split 1/3 to a flame ionization detector (FID) and 2/3 to the mass spectrometer through a jet separator. Compound quantitation was accomplished by digital integration of the FID response. The FID-peak area response was based on a 113 ppm hexane standard prepared by Matheson Gas Products. Quantitation of NDMA was based on an absolute calibration curve for this compound prepared by serial dilution of a 1% standard.

Identification of unknown compounds was accomplished by the data system based on comparison of fragmentation patterns with a data library containing approximately 23,900 compounds. The MS ionization chamber was maintained at 250 C and the source pressure at 10^{-6} Torr or less. The data system was calibrated between mass-to-charge (m/e) 12 and 219, with the mass spectrometer scanning repetitively at 2 seconds per decade. Data acquisition was done at 10 kilohertz with an ion threshold of 1.

Cryotrap Analysis

The analytical procedure for the cryotrap samples is shown schematically in Figure 6. Each of the three cylinders constituting a sample set was analyzed individually, first by direct injection, and then by concentrated injection via cryogenic sample loop. This procedure was necessary to determine sensitivity settings and a concentration multiplier for calculation of the air sample concentration. The detailed procedure was as follows: Each cylinder was heated to 135 C to revaporize the trapped material and assure a homogeneous sample. The pressure of the bottle was recorded. For direct analysis, the sample was expanded into a 3 cc preevacuated sample loop, equilibrated at room temperature, and valved into the GC with helium carrier gas. For the concentrated analysis, the 3 cc sample loop was evacuated and immersed in liquid nitrogen. The content of the cylinder was then bled slowly (6 cc/min) into the sample loop until equilibrium was established. Following sample concentration, the loop temperature was raised slightly (to above -183 C) and opened momentarily to release any trapped oxygen. The loop was then closed, heated to 135 C, and valved into the GC.

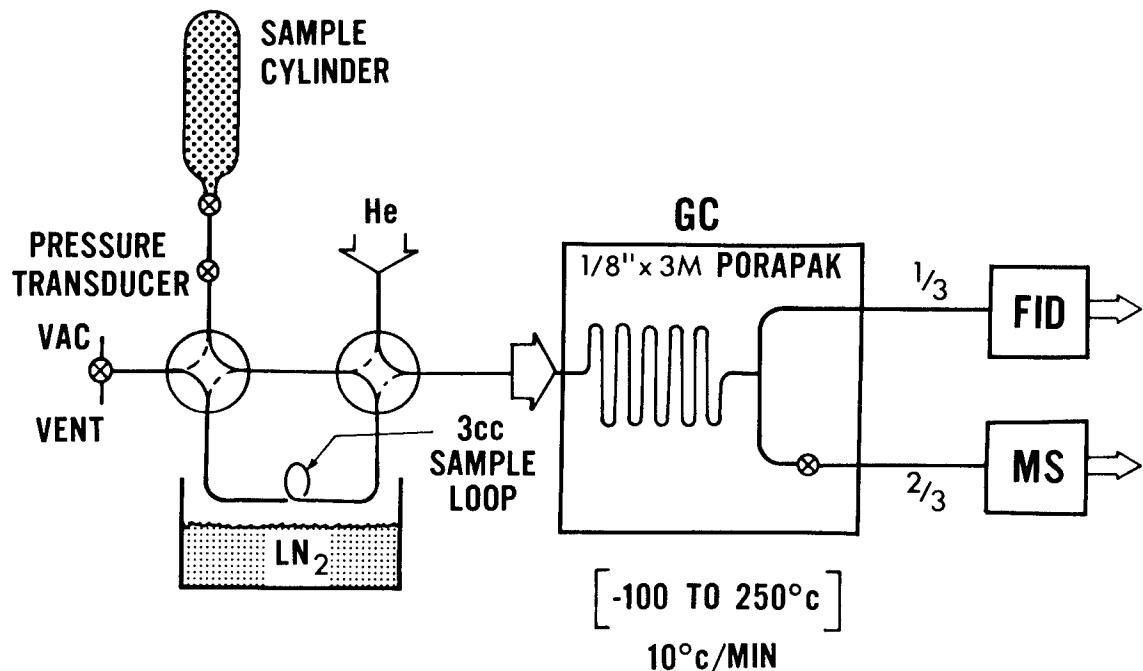


Figure 6. Schematic of analytic procedure employed with cryotrap and Tenax-GC sampling systems.

The concentrated analysis from each of the three cylinders was condensed into a single report of the individual compounds contained in the cryoset as follows:

$$C = \frac{M_1 + M_2 + M_3}{(v)(t)}$$

where

C = concentration of compound in original air sample, $\mu\text{g}/\text{m}^3$
 M₁ = mass of compound in 0 C trap, μg
 M₂ = mass of compound in -78 C trap, μg
 M₃ = mass of compound in -175 C trap, μg
 v = sample flow rate, m^3/min
 t = sample time, min.

The mass of each compound contained in the individual traps was calculated as follows, using the 0 C trap as an example:

$$M = m F \left(1 + \frac{153.6 P_0}{3.0 P_1}\right)$$

where

m = micrograms of individual compound obtained from FID peak area (Autolab calibration based on a hexane response or NDMA calibration curve)
 F = concentration factor
 P₀ = cylinder pressure (Torr) before analysis
 P₁ = loop pressure (Torr) of initial unconcentrated analysis
 153.6 = combined volume (cc) of cylinder, sample loop and connections
 3.0 = volume of sample loop (cc).

Similar equations applied to the -78 C and -175 C traps.

Tenax-GC Analysis

Analysis of the Tenax-GC cartridges was similar to that of the cryotrap cylinders except that only a concentrated GC run was conducted. The Tenax cartridge was thermally off-gassed into the 3 cc GC sample loop at liquid nitrogen temperature. Heating of Tenax cartridge was accomplished by a hinged aluminum manifold containing two 300 watt heaters. Offgassing was initiated with

the cartridge at room temperature with helium flow at 30 cc/min. The cartridge was then heated to 240 C in 10 minutes and maintained at 240 C for an additional 10 minutes with continuous helium flush. The sample loop was then closed, removed from liquid nitrogen and wrapped in a heating blanket. Following 10 minutes of equilibration at 115 C, the loop contents were valved into the GC with a one minute injection. The GC-MS operating conditions were identical to the cryotrap analysis.

Thermal Energy Analyzer

The coupled GC thermal energy analyzer (Thermoelectron Model TEA-502) has been described in detail by Fine et al. (1975b). A schematic of the instrument is shown in Figure 7. The TEA was specifically designed for analysis of volatile nitrosamine compounds and operates on the principle of pyrolytic cleavage of the heat labile nitrosyl group (NO), which it detects by chemiluminescent reaction with ozone. A cold trap serves to condense organic residue following pyrolysis. The instrument consists of five components: gas chromatograph; catalytic pyrolyzer; cold trap; chemiluminescent reaction chamber; and photomultiplier detector.

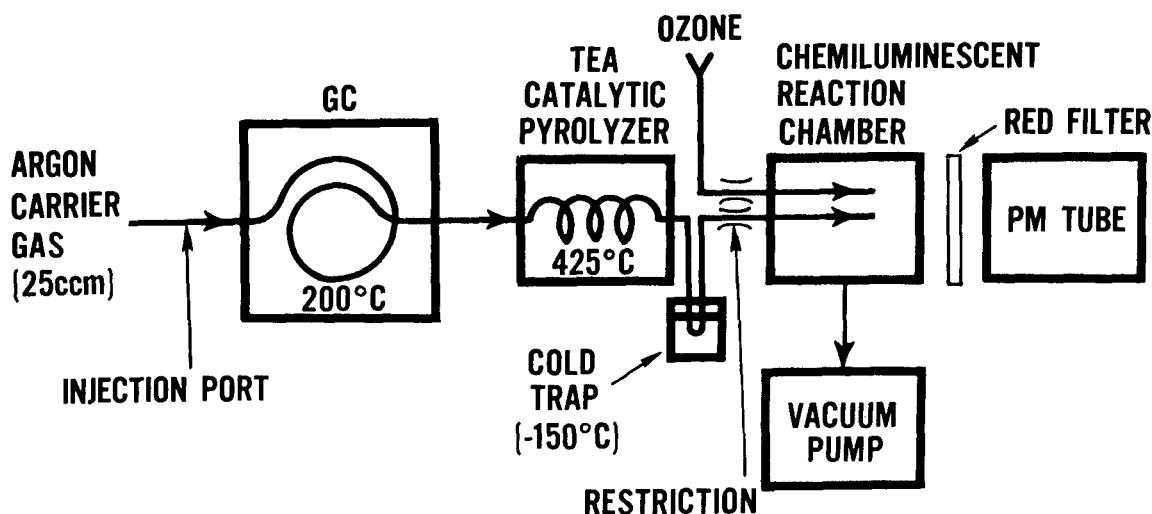


Figure 7. Flow schematic for GC-Thermal Energy Analyzer (GC-TEA).

The chromatographic column supplied with the instrument was a 1.5 m long x 2 mm I.D. stainless steel tube packed with 15% free fatty acid phase (FFAP) on Chromosorb W (80-100 mesh). The carrier gas was Argon at a flow rate of 25 cc/min. The GC was operated isothermally at 200 C. The catalytic pyrolyzer was maintained at 425 C, and the cold trap at -150 C (the latter was accomplished by means of the third sample cylinder in a 3-stage cryoset).

Sample preparation for the GC-TEA was done by extraction and volumetric concentration of the bubbler contents. Each combined sample of 200 ml KOH solution was extracted three times with 100 ml Analytical Reagent (AR) grade dichloromethane (DCM). The extracts were combined and placed in a rotary vacuum evaporator (Buchi Model Rotavapor-R) with the flask in a 50 C water bath. When the column of DCM was reduced to approximately 5 ml, one ml of AR benzene was added, and the evaporation continued until approximately one ml remained. In a later modification AR iso-octane was substituted for benzene. In either case, the final volume was recorded and concentrated sample analyzed by TEA. Standard solutions of 1 and 5 mg/liter NDMA in water were similarly extracted and concentrated to determine recovery efficiency. The TEA was calibrated with standard solutions (1 to 10 mg/liter) of NDMA in iso-octane. Calculation of air concentrations of unknowns was made according to:

$$C = \frac{100 C_a V_a}{E v t}, \text{ where:}$$

C = NDMA concentration in chamber, $\mu\text{g}/\text{m}^3$
C_a = NDMA concentration in extract, $\mu\text{g}/\text{ml}$
V_a = final volume of extract, ml
E = extraction efficiency (50%).

RESULTS

Two separate comparison studies were conducted in the toxic hazards exposure chamber, the first in April, 1976 and the second in June, 1976. Each study employed atmospheres of background chamber air, 5 ppm pure UDMH, and 5 ppm UDMH spiked to 1200 mg/liter with NDMA.

APRIL 1976 TEST

The sampling schedule for the April 1976 study is shown in Figure 8. The background cryoset was taken on 27 April and the background bubbler and Tenax-GC cartridges were taken on the morning of 28 April prior to initiation of the pure UDMH run. The cryoset samples were 4 hours in length (72 liter); the bubblers were 60 minute (300 liter), and the Tenax tubes either 60 minute (60 liter) or 90 minute (90 liter). The cryoset and bubbler samples for the UDMH runs were sequential replicates. The Tenax cartridge samples were both parallel and sequential.

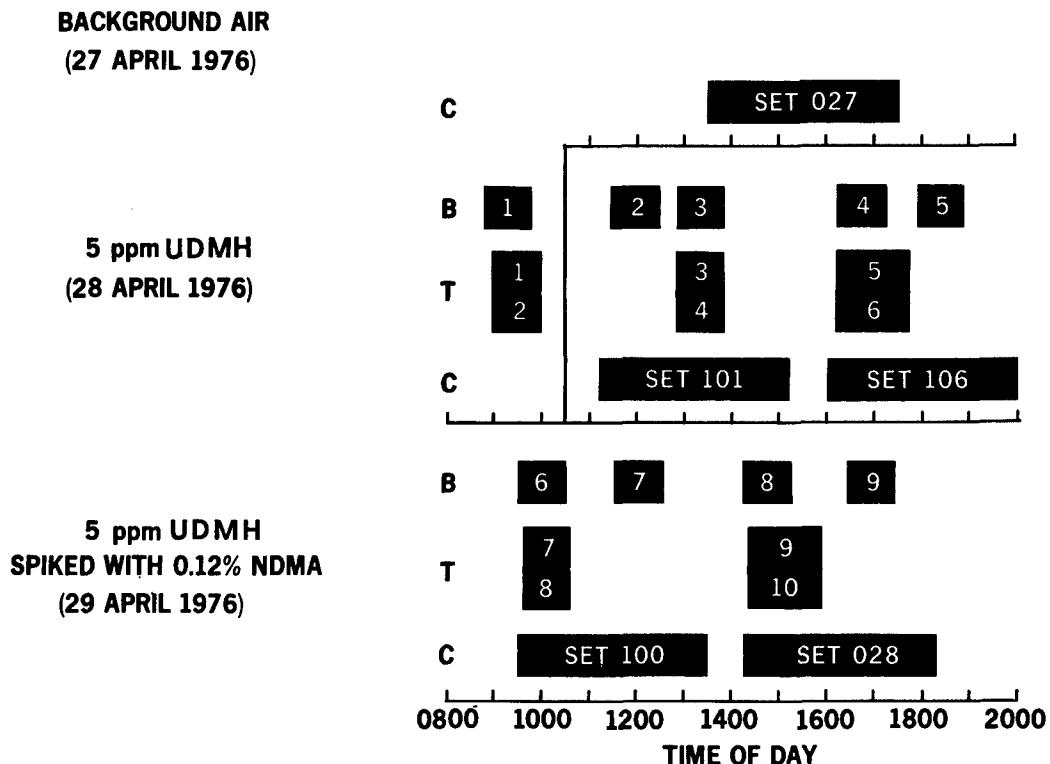


Figure 8. Sampling schedule for April, 1976 test. C-cryotrap sample; B-bubbler sample; T-Tenax-GC cartridge sample.

The NDMA results for the April, 1976 sampling study are shown in Table 1. As expected, the background air samples showed negative in NDMA. Rather unexpected, however, was the positive identification of NDMA in the 5 ppm "pure" UDMH run, which was confirmed by both Tenax-GC and TEA procedures. The finding of NDMA in the spiked UDMH test was expected, although the levels were almost twice the calculated concentration of 19 $\mu\text{g}/\text{m}^3$. Agreement between the Tenax-GC and TEA methods in both tests was within the limits of experimental error for trace level work. The Tenax samples appeared to be somewhat more reproducible than the TEA procedure which may be a reflection of extraction variability in the latter. The difference between the average NDMA concentration in the pure and spiked test was not statistically significant for either of the Tenax-GC cartridges or the TEA procedures.

TABLE 1. NDMA TEST RESULTS ($\mu\text{g}/\text{m}^3$)
AMRL EXPOSURE CHAMBER (APRIL, 1976)

<u>Chamber Atmosphere</u>	<u>Cryotrap</u>	<u>Tenax</u>	<u>TEA</u>
Background Air	ND ¹	ND ND	ND
5 ppm pure UDMH	ND ND	Lost (29.8) ² 21.3 <u>(22.0)</u>	49.6 17.6 13.0 <u>34.0</u>
Mean		24.4	28.6
S.D.		4.7	16.7
5 ppm UDMH spiked NDMA (0.12% v/v)	ND ND	22.7 23.8 37.5 <u>24.1</u>	19.8 14.0 55.4 <u>57.8</u>
Mean		27.0	36.8
S.D.		7.0	23.1
Lower Detection Limit	2.8 (72-liter)	3.3 (60-liter)	0.2 (300-liter)

¹Not detected

²Identified by mass spectrometer as trimethylhydrazine.

Of some concern to us was the occasional identification of NDMA by the mass spectrometer as trimethylhydrazine (TMH). These cases are identified parenthetically in Table 1. Both TMH and NDMA have a molecular weight of 74 and exhibit similar fracture patterns. We believe this identification was anomalous, due in part to incomplete separation of the NDMA peak from UDMH, and in part to the extremely low concentration of compound available for analysis. Figure 9 shows the mass chromatogram for Tenax cartridge no. 6 where NDMA was identified as trimethylhydrazine, compared to cartridge no. 9 where NDMA was identified as such. Designation of the questionable peak (Figure 9a) as NDMA appeared warranted based on similarity in elution time, correspondence of m/e 74 peak, and the concomitant finding of NDMA by the TEA procedure.

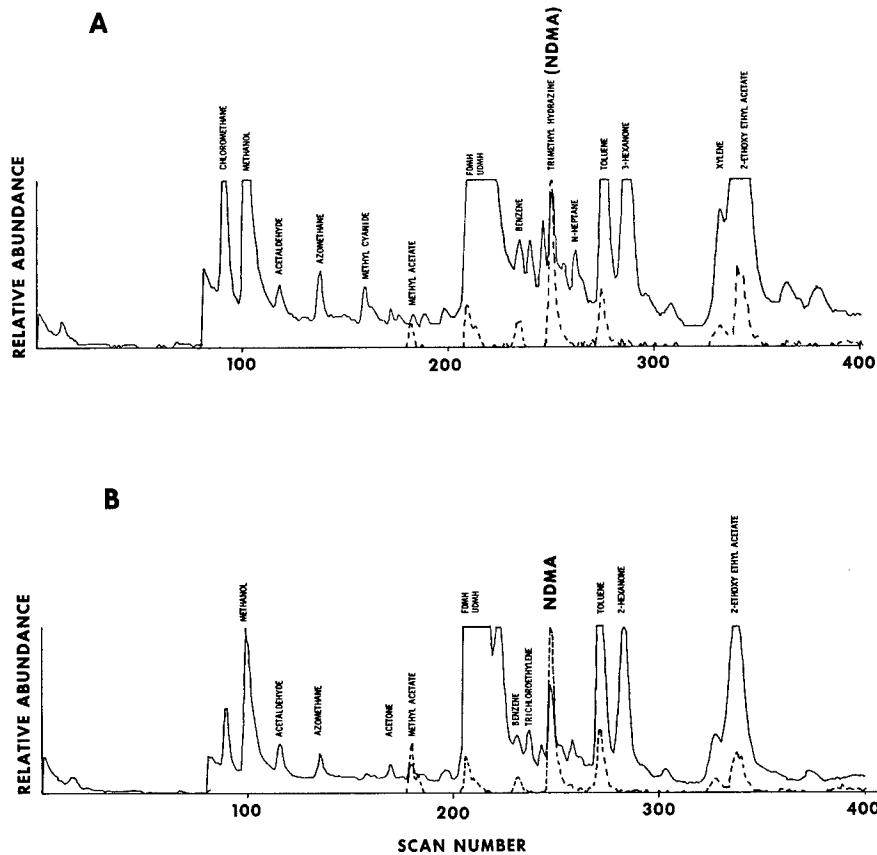


Figure 9. Mass chromatogram of Tenax-GC cartridges from April 1976 test series; solid line-total ion count; dashed line-m/e 74 ion count; A-Tenax sample no. 6 (NDMA identified as trimethylhydrazine by the mass spectrometer-data system); B-Tenax sample no. 9.

The negative cryotrap results in both the pure and spiked UDMH tests was attributed to inadvertant misconnection of the sampling line. Not only was there no detection of NDMA in any of the cryotrap sets, but neither was there any evidence of UDMH or its breakdown products. This fact, coupled with the unexpected finding of NDMA in the pure UDMH test, prompted a repeat of the study.

JUNE 1976 TEST

The sampling schedule for the repeat test, conducted in June, 1976 is shown in Figure 10. This protocol was essentially identical to the April test except that all background testing was completed on the first day.

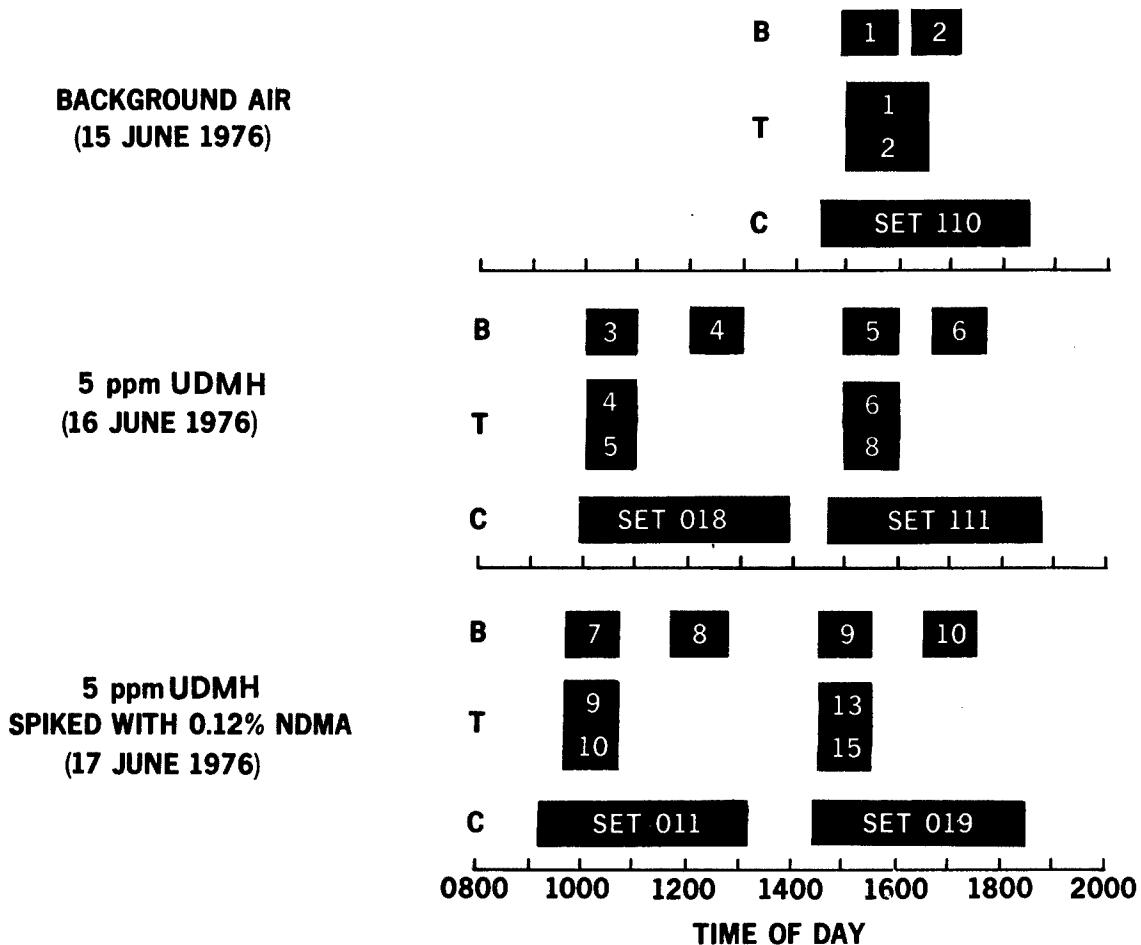


Figure 10. Sampling schedule for June 1976 test.

The NDMA results from the June 1976 test are shown in Table 2. The analyses again showed NDMA in both the pure and the spiked UDMH tests, in varying concentration, but this time by all three methods. Agreement between the Tenax and TEA procedures was within the limits of experimental error. The difference between the NDMA concentration in the pure and spiked runs, although somewhat less variable than the first test, was not statistically significant.

TABLE 2. NDMA TEST RESULTS ($\mu\text{g}/\text{m}^3$)
AMRL EXPOSURE CHAMBER (JUNE, 1976)

<u>Chamber Atmosphere</u>	<u>Cryotrap</u>	<u>Tenax</u>	<u>TEA</u>
Background Air	ND ¹	ND ND	(17.8) ² (17.3)
5 ppm UDMH	6.1 8.0	51.4 24.1 43.5 <u>38.6</u>	39.3 46.2 40.1 <u>33.5</u>
Mean		39.4	39.8
S.D.		11.5	5.2
5 ppm UDMH spiked with NDMA (0.12% v/v)	7.4 7.6	51.3 50.1 Disc ³ <u>51.0</u>	44.2 51.3 37.5 <u>64.4</u>
Mean		50.8	49.4
S.D.		0.6	11.5
Lower Detection Limit	2.8 (72-liter)	3.3 (60-liter)	0.1 (300-liter)

¹Not detected

²Elution time contraindicative of NDMA

³Discarded due to flowmeter malfunction.

The observation of a positively responding compound in the TEA analysis of background air samples was believed anomalous. This compound was reproduced in both samples and eluted at approximately 180 seconds, whereas NDMA elution in the prepared standards was uniformly in the range from 145 to 160 seconds. An attempt to identify the unknown compound by GC-MS analysis of the background extract was unsuccessful owing to insufficient concentration. One possible explanation for background artefact was that the impinger collected samples remained frozen at dry ice temperature for approximately 12 weeks awaiting transit and setup of the TEA instrument in our laboratory. GC-MS analysis of both the cryotrap and Tenax collected background air showed traces of ketones and esters (paint offgassing) which could have degraded to formaldehyde or some other TEA responding compound. TEA analysis of the unaerated KOH was negative.

The low NDMA concentration observed in the cryotrap samples was probably due to low recovery efficiency. As reported earlier, the temperature used to revaporize the trapped material was limited to 135 C by the valve seating material. This limit was below the boiling point of NDMA and thus precluded total removal of NDMA from the sample cylinder.

ANALYSIS OF LIQUID UDMH

In order to determine the source of NDMA found in the pure UDMH atmospheres, NDMA assays were conducted on both the pure and spiked UDMH liquids used in the June test. Multiple analytical methods were employed including GC-TEA, GC with alkali flame ionization detection (GC-AFID), and mass spectrometry. The results, listed in Table 3, were surprising in both their magnitude and agreement. All three analytical methods showed substantial concentrations of NDMA in both types of UDMH. Although the spiked UDMH assayed uniformly higher in NDMA than the pure, none of the methods detected a 1200 mg/liter difference between the two. This nonconfirmation of the known NDMA spike remains unexplained but may have been due to air oxidation as discussed later.

TABLE 3. NDMA ANALYSIS (mg/liter) OF LIQUID UDMH SAMPLES

<u>Method</u>	<u>Pure UDMH</u>	<u>Spiked UDMH</u>
TEA	1015	1250
GC-AFID	1020	1460
Mass Spectrometer (m/e 74 ion count)	710	980

Of the three UDMH analysis methods, we had more confidence in the TEA because of its specificity and reproducibility. One question of importance was the relative sensitivity of the TEA to UDMH itself, compared to NDMA. UDMH detection by TEA could not be excluded *a priori* because chemiluminescent analysis of UDMH has been shown in both the direct and indirect mode via reduction pyrolysis (Luskus and Kilian, 1976). Figure 11 shows the response of the TEA to 10 μ l injections of several UDMH and NDMA samples. Comparison of Figure 11a and 11b shows the relative response of UDMH and NDMA at the same concentration (1 mg/liter) in isoctane. UDMH gave no response. Figures 11c and 11d show the response of the same UDMH material (Aldrich) in pure form (11d), and at a concentration of 1000 mg/liter in isoctane (11c). The NDMA concentration of the two samples was determined to be 270 and 0.78 mg/liter, respectively, based on peak height of NDMA. The calculated NDMA concentrations (for 11c based on the measured value for 11d, and vice versa) are shown parenthetically beside each peak. The conclusion was that any UDMH response which may have obtained at high instrument sensitivity (low attenuation) was effectively filtered at the lower sensitivity setting required for NDMA analysis of liquid UDMH. Comparison of 11b and 11c shows that the TEA is at least 1000 times more sensitive to NDMA than UDMH. As a further comment, we have used the TEA to analyze UDMH from various sources, and found NDMA concentrations ranging from 10 to several hundred mg/liter. In a recent Air Force interlaboratory UDMH correlation sample, TEA analysis gave an NDMA concentration of 128 mg/liter; the average of five AF laboratories was 112 mg/liter with a standard deviation of 37 mg/liter. Hence, the accumulating data base on TEA analysis of UDMH supports the specificity of the instrument for nitrosamine detection.

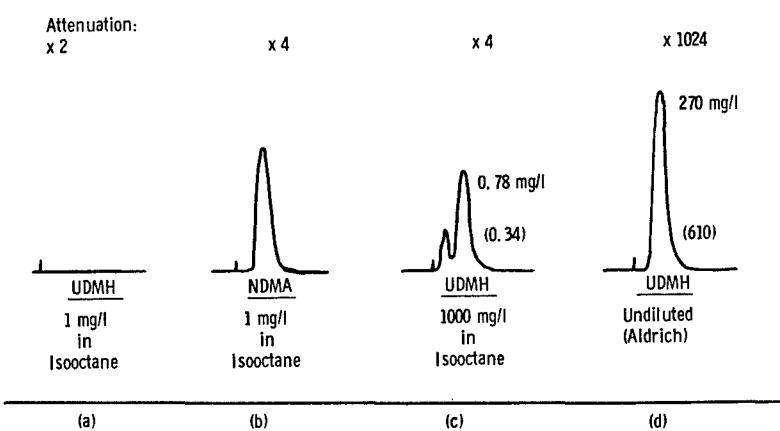


Figure 11. TEA response to UDMH and NDMA.

The GC-AFID analysis of UDMH was less reliable because the NDMA appeared as a small peak on the tailing side of UDMH. The quantitation shown in Table 3 was based on the product of peak height and peak width at one-half the peak height, against a 1000 mg/liter NDMA standard in isoctane. The GC-AFID analysis was conducted isothermally at 200 C using a 3.7 m x 3.2 mm O.D. column packed with Porapak Q (100/120 mesh). The carrier gas was helium at a flow rate of 30 cc/min. Under these conditions, NDMA eluted at approximately 350 seconds.

Mass spectrometer analysis of UDMH was done by comparing the m/e 74 ion count in the pure and spiked UDMH samples against water standards of 500 and 1000 mg/liter NDMA. One microliter of each sample and water standard was injected directly into the batch inlet, and the ion count averaged over 15 scans. The batch inlet temperature was 175 C and the source temperature was 250 C. The principal uncertainty in the MS analysis appeared to be the variation in the number of m/e 74 ions between scans which may have been due to variable source pressure. The DuPont 21-491 mass spectrometer was designed primarily for qualitative analysis and is not equipped with appropriate control equipment for accurate quantitative work.

DISCUSSION AND CONCLUSIONS

The test results supported one conclusion and raised several questions. On the basis of reproducibility, the Tenax-GC and impinger/TEA methodologies for sampling/analysis of NDMA-in-air appeared superior to the cryotrap. The agreement between the Tenax and TEA methods was acceptable for trace level analysis and corresponded to that found by Fine et al. (1976b) and Pellizzari et al. (1976b) in previous studies. We are currently replacing the valves on the cryotrap to permit revaporization at higher temperatures, and thus improve the recovery efficiency.

The principal questions resulting from this study were the finding of NDMA in the "pure" UDMH atmospheres and the nonconfirmation of a 1200 mg/liter "delta" between the pure and spiked UDMH liquid samples. On a relative basis, the liquid UDMH analyses supported the chamber air findings. In both the atmospheric and liquid samples the NDMA concentration in the spiked UDMH test was approximately 25 percent greater than the pure. The overall agreement in the atmospheric concentrations measured in both the April and June tests (by two methods) supported the presence of

NDMA in the chamber. On an absolute basis, however, the atmospheric concentrations appear high. Assuming complete vaporization of injected UDMH liquid, chamber air containing 5 ppm (12.5 mg/m³) of spiked UDMH should have contained 19.1 µg/m³ (6.3 ppb) of NDMA when the UDMH was spiked to 1200 mg/liter. Our measurements showed average chamber air NDMA concentrations in the range from 24 to 40 µg/m³ with pure UDMH, and 27 to 50 µg/m³ with spiked UDMH. Based on our UDMH analysis (by TEA), these values were 50 to 150 percent greater than theoretically expected. Several explanations may obtain. In practice, the rate of liquid UDMH injection was somewhat greater than that calculated to maintain a chamber concentration of 5 ppm owing to transfer and surface losses. Because NDMA is less reactive than UDMH, it might be expected to more readily traverse the inlet system and therefore appear in the chamber in greater concentration. This factor, although active, probably did not account for more than 10-15 percent of the NDMA elevation at steady state. A second and more important possibility is the potential for air oxidation of UDMH to NDMA. Evidence for this reaction has been reported (Urry et al., 1965), but the NDMA formation rate has not been established. A third possibility, of course, is that air oxidation of UDMH occurred as an artefact following sampling, transport and/or in the analytical procedures. Although Fine et al. (1976b) and Pellizzari et al. (1976b) have exhaustively looked for NDMA artefact from dimethylamine precursor, little work has been done on NDMA formation from UDMH. While the formation of similar artefact levels in two entirely different sampling-/analytical procedures appears unlikely, this factor cannot be ruled out entirely.

The reason for nonconfirmation of a 1200 mg/liter "delta" between the pure and spiked UDMH liquid samples remains largely subject to conjecture. The most probable explanation is liquid state oxidation resulting from air contact. The NDMA content of liquid UDMH is known to increase with air exposure. Cursory experiments in our laboratory have indicated that the rate of increase may be as high as 10 mg/liter per hour in a static liquid exposure and much higher in aerated samples. It should be mentioned that because of instrument transfer, our UDMH analyses were delayed approximately 12 weeks after the June, 1976 chamber study. Hence, despite the precaution of nitrogen blanket storage, we cannot be certain that the liquid UDMH samples remained uncontaminated in preparation or transit.

The results of these studies suggest a need for further experimentation to quantitate the rate of air oxidation of UDMH in both the liquid and gaseous state. Previous studies on the auto-oxidation of UDMH (Urry et al., 1965) dealt with the disappearance of UDMH and the appearance of its principal oxidation products, formaldehyde dimethylhydrazone, ammonia, and dimethylamine. Although NDMA was reported in "trace" quantities (by infrared detection), its significance at that time was not apparent. The current state of knowledge on the toxicity of NDMA and the advent of more sensitive NDMA detection methods warrant further investigation into the possible formation of NDMA from UDMH.

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OPEN FORUM

DR. PHALEN (University of California, Irvine): Dr. Taylor, you mentioned that the HCl effects on plants can be greater at high humidity than at low humidity. Could you speculate on the reasons for that?

DR. TAYLOR (University of California, Riverside): I can speculate but I don't know. With relative humidity above 50%, we would expect stomates to be open more widely which would allow the entrance of the HCl. Also with this higher humidity, I suppose there might be even more moisture around the tissue of the leaf that would encourage HCl injury. But I think probably most of the reason for a greater effect at high humidities is that the leaf is more turgid, stomates are open, and it is capable of absorbing more of the material.

CAPT. LIND (6570 Aerospace Medical Research Laboratory): When you have been out in the field observing plant damage because of HCl exposure, have you ever noticed any nonvascular plants that have been damaged?

DR. TAYLOR: I have noticed injury to fruiting bodies of mushrooms but only when the exposure was to an HCl aerosol. Otherwise, I have not seen any damage in nonvascular plants. There is the possibility of HCl affecting the relationship of mycorhiza with plant roots. Many of the plants, most plants in fact, do much better if the roots are infected with a fungus called mycorhiza which gives a greater surface area and more opportunity to pick up nutrients from the soil. In most cases, there is a very delicate balance between the mycorhiza and the plant. We are doing some work to see if injury from HCl to the plant will reduce the mycorhiza. One of the preliminary tests indicated that injury from ozone did greatly reduce the amount of mycorhiza in plants.

DR. INMAN (6570 Aerospace Medical Research Laboratory): When you were looking at these specific accidental incidents, did you notice any obvious difference in sensitivity of different species of plants?

DR. TAYLOR: Yes, there's a big difference, not only between species, but between varieties of the same ones. This is why I mentioned the mission variety of almond because it is highly susceptible and others are not. There are lists of plant species tabulated according to susceptibility as susceptible, intermediate, and tolerant species. I hesitated to present that information because it's still considered controversial. You will find some researchers that will list one plant as a tolerant plant and another researcher finds that it's susceptible or intermediate. One example of this problem is the sunflower which is listed by some as being intermediate to susceptible. In Texas, where the sunflower is quite prominent, I have found it to be quite resistant compared to many of the other types of vegetation in the area. There is a wide difference in susceptibility of species.

DR. BERRY (6570 Aerospace Medical Research Laboratory): You've shown a lot of morphological damage due to these phyto-toxicants. Do you have any evidence of physiological response in plants that have been exposed to HCl and chlorine such as changes in photosynthesis or respiration?

DR. TAYLOR: We ran some studies of that about 8 or 10 years ago but as near as we could tell, the interruption of photosynthesis was about equivalent to the destruction of tissue. Dr. Indres, one of our colleagues, is doing some electron microscope studies on tissues exposed to HCl and he is finding symptoms of injury within the cell very similar to ozone. He is finding crystallization in the stroma of the chloroplast which had been reported from ozone exposures maybe 10 or 15 years by Thompson. This crystallization is thought to be caused by condensation of an enzyme in the chloroplast. It's also interesting that in some leaves he will get crystallization and then 12 hours later, those chloroplasts have recovered and the crystallization has disappeared. He also finds breakage in the plasma membrane. He's not sure whether it's a direct breakage in cell wall or whether it's an effect on the cell wall which makes it brittle so that in sectioning, his knife breaks out pieces of the cell wall. We don't know for sure about that yet, but there is a breakage in cell wall, breakage of the membrane, and crystallization in the chloroplast. That's what we've seen so far.

CAPT. LIND: I'd like to make one comment related to Dr. Berry's question. Dr. Norman LeCass and Thaddeus Godish at Penn State did some studies on photosynthesis in tomato plants

that had been exposed to HCl and one of their findings was that at concentrations below the threshold for visible plant damage, they found changes in the photosynthetic rate and in respiration and transporation. The respiration and transporation rates were increased and photosynthesis, I believe, was reduced. There may well be some physiological changes taking place in the plants even though there are no visible plant damage symptoms.

DR. TAYLOR: I believe that effect was measured during the time of fumigation and it didn't persist very long after they took the tomato plants out of the fumigation chamber. Isn't that right?

CAPT. LIND: I think that's correct. The dissertation was written by a man named Thaddeus Godish who was at Penn State about 3 or 4 years ago. It's unpublished but his dissertation is available.

DR. TAYLOR: I'm familiar with that report. I've talked with him and we've found a similar type thing. We didn't know really whether this was an effect of the HCl in causing stomates to close or partially close or whether it was a physiological effect on the tissue. We kept our nondispersive infrared measurement going and soon after we took the plants out of the fumigation, they were back up to the original level of photosynthesis but only if we did not have visible symptoms develop on the leaves. We haven't explored that as far as we should yet.

DR. SLONIM (6570 Aerospace Medical Research Laboratory): Dr. Granett, did I understand you to say that the zinnia plant was the only one that had an inverse relationship between extent of damage and age? I wasn't sure whether you explained that on the basis that plants on the upper side of the abscissa were very old. Do you have some explanation for that?

DR. GRANETT (University of California, Riverside): The zinnia plant was the only one that I discussed that we didn't do at a single age period. All the other plants were run at a particular age. We grew a population of zinnia plants and took samples at about weekly intervals from that population, gave them a single exposure, and looked for damage. In this experiment, we felt that the older the plant was when it was exposed, the less it was damaged. But, of course, 41 days is not an old plant as such. It's the oldest seedling that we looked at.

DR. SLONIM: So you are not saying then that there was really a true inverse relationship if the older plants are not as old as you are saying they are. In other words, you had a very high correlation there and I was surprised to see for the first time this morning that the older plants essentially show less injury damage than the younger ones on your curve.

DR. GRANETT: That is what we found.

CAPT. LIND: When Dr. Taylor showed his slides this morning, he indicated that the older leaves were the most susceptible. In the same respect with your zinnias, you indicated that the older leaves were the least susceptible. Are there species difference- generally or is there any general trend?

DR. GRANETT: There is no general trend that I can define for sure at this time. I believe that there are probably species differences. But in these plants we found that the more mature leaves seemed to be more tolerant.

CAPT. LIND: Do you know of any morphological or physiological reasons for differences in susceptibility of an aged leaf of one species compared to another?

DR. GRANETT: None that I'd like to speculate upon at this time.

MR. VERNOT (University of California, Irvine): This is addressed to Dr. Miller and it's more in the nature of a comment rather than a question. Your interpretation of the figures seemed to indicate that there was some oxidation of the UDMH in the chamber atmospheres to dimethylnitrosamine. Initially, however, you mentioned that we had found out that our UDMH was contaminated with .12% of the dimethylnitrosamine after we had begun the exposure of the animals to that particular material. That wasn't really true. We knew the material contained impurities to that extent prior to the start of the experiment. However, at that time, there were two factors which caused us to go ahead with the experiment. One was that this was the kind of material that men were being exposed to if exposure took place and the other was that OSHA has just promulgated its list of carcinogens which included nitrosodimethylamine. They had also said that if the material contained less than 1% of the carcinogen, the mixture was not to be considered a carcinogen. Therefore, we went ahead with the study. That's just a clarification. Concerning the possibility of the air oxidation of the UDMH to nitrosamine, I think that there is enough uncertainty in

some of the results that you got such as your finding significant amount of the dimethylnitrosamine in a chamber that contained no UDMH or nitrosamine, and the fact that when we conducted the study that Mr. Haun reported on that we did not see liver injury when we exposed dogs to purified UDMH. In this study, we attempted to unravel the cause of the liver effects which we had seen, the elevated SGPT values. And at that time, when we used what we thought and what was analyzed in the AMRL analytical facilities as pure UDMH, we did not get the liver effects which we had gotten in the initial study. When we spiked the material with 0.12% dimethylnitrosamine, we got the very same liver effects which we had gotten in the earlier study. This indicated to us two things on a biological basis. One was that the UDMH we were using in the second study was reasonably pure and not contaminated with dimethylnitrosamine. Secondly, that the dimethylnitrosamine was not being formed in the chamber because we would have found the effect with the pure UDMH exposure. So on a biological basis, I think there is some evidence which indicates that if there is any oxidation of UDMH to dimethylnitrosamine, it isn't significant.

DR. MILLER (U.S. Air Force School of Aerospace Medicine): I did not address the issue of the biological results versus what we got because I don't have any information, all I have is speculation. And that is that the material, indeed, that you used may well have been relatively pure. As I understand it, those experiments were done in the early part of this year which complicates the issue. The only comment I was going to make was that we know that the nitrosamine content of UDMH will increase in the liquid state if UDMH is exposed to air. What we recently found out is that that increase can be quite large and it can increase quite fast on the order of, let's say, 10 to 30 mg/liter per hour if the material is openly exposed to air. The samples which we took back with us were not completely under a nitrogen blanket all the time and we may be seeing or reporting on increases which occurred even between the time we completed our experiments in April and June. Now, the analysis on these samples we have were just done last week. I don't know what the storage conditions of the UDMH were between the time that you did the dog exposures and we did these last test runs. That may be critical to try to determine. I would make one further point and that is that I think that the standard method for doing NDMA in almost everything is going to be the TEA. I didn't start out feeling this way but this is what I believe now.

DR. BACK: I have a statement rather than a question in response to Dr. Miller's statement that UDMH deteriorates very, very rapidly on standing. If that's the case, then all of the work that we've done since 1960 certainly doesn't show it. I had a high suspicion in 1960 that UDMH went to n,nitrosodimethylamine in the body or while sitting around on the shelf. If you take pure UDMH and put it on a shelf and open it to air, it turns yellow. We looked for n,nitrosodimethylamine in that kind of situation and were unable to identify it analytically. We've also done subacute and chronic, subchronic testing with this kind of UDMH material in rats, monkeys, dogs and all kinds of animals. Not once have we ever seen true liver damage.

N,nitrosodimethylamine is one of the most potent hepatotoxins that I know of. To make matters worse, we just completed very recently studies in mice using n,nitrosodimethylamine and got a dose response curve on hepatotoxicity and if you look in the literature, everybody that's ever used the compound uses it in doses starting at milligrams per kilogram whereas the hepatotoxicity started out at doses of micrograms per kilogram. Over the past 15 years I have conducted studies on UDMH that we got from Kodak and from Aldrich and from other suppliers although it probably all came from the same manufacturer and was repackaged. I should have been seeing hepatotoxicity from UDMH if it were contaminated with dimethylnitrosamine to any great extent. And I have not seen that. Now that makes me highly suspicious of what indeed this TEA instrument is measuring. It makes me highly suspicious also because this isn't the first time we used UDMH in a long-term continuous exposure of animals. We did it before and we didn't see hepatotoxicity. Why suddenly are we finding tremendous amounts of n,nitrosodimethylamine in it? There's something that is very suspicious about the large amounts that you are finding. Besides that, you didn't find any in the cryotrap. Or if you did, there was no difference between the so-called unspiked and the spiked. And that bothers me, the fact that you can't add your figures together and come up with a reasonable answer on the TEA or on your GC. You're measuring something that I feel may well not be nitrosodimethylamine although there are certain indications that it might be. Biologically, it isn't there in any great amount.

DR. MILLER: I agree. There are 2 or 3 aspects to the data which just don't track. I would defend myself on just one point. I did not say that UDMH degrades when it sits around, I said that if it has any contact with air, the nitroso content as measured by the TEA will increase. I would like to determine exactly what this is. If we could, it would be useful for Air Forces purposes to be able to show that the TEA is, indeed, measuring something

else and calling it nitrosamine. If we could have been able to do that, and that's what we originally started out to do and are really attempting to do now, we'd be in a little bit better position to defend ourselves.

DR. A. THOMAS (6570 Aerospace Medical Research Laboratory): Dr. Back, you remember when we did intraperitoneal LC₅₀'s with UDMH which was kept in contact with air and repeating it, I think every other week for 6 weeks. I believe you didn't see any significant increase in the toxicity of the material.

DR. BACK: In our very early work we took UDMH, put it in water and let it deteriorate and then we did LD₅₀'s using that compound as it deteriorated and it got more yellow and more yellow. As I can remember, the LD₅₀ for mice was around 125 mg/kg. At the end of 12 days, it did become more toxic. And the LD₅₀ went from 125 down to something like 75, if I can remember off the top of my head. It's many years ago. Then as we got to 27 days, the toxicity almost completely disappeared. If indeed all of the UDMH went to n,nitrosodimethylamine in water, it should have increased the toxicity a hundred fold or a thousand fold but it didn't. This was done in water which was also exposed to air and light which are beautiful ways of oxidizing the compound. We never did see a conversion to DMNA but I don't know what it is we are seeing.

MAJOR MAC NAUGHTON (Envirionics Directorate, U.S. Air Force Civil Engineering Center): We've looked at the oxidation of UDMH. We got involved when this initial problem started looking at what would happen if you would take solutions that contain UDMH and lagoon them. These were primarily caustic solutions that were waste from the FMC plant which produces UDMH. We found that UDMH is very readily photolyzed with ultraviolet sunlight. I have done the same thing that Dr. Miller has, looking at the production of a peak that comes out the same place on the gas chromatogram and that is identified with a mass spectrometer and has the same mass spectrum that we get for n,nitrosodimethylamine. You can increase the amount you get by letting it sit out like Dr. Miller did or very dramatically by bubbling air through pure UDMH. You can get very high concentrations of whatever this is. We say it's DMNA. Whether it is or not maybe it can be left up to question. It does break down under ultraviolet. We expect that since it adsorbs energy around 3200 nanometers that that's probably where the main spectrum is that causes the breakdown.

MR. GEIGER (6570 Aerospace Medical Research Laboratory): I would like to make a comment concerning some very recent analyses I made and I've already discussed these with Dr. Miller. Early this week I was asked to analyze two samples of UDMH by the UCI contractor. They were given to me by Mr. Pollard of UCI. One sample was the pure UDMH which was stored for over a year in a brown bottle. The other sample was the UDMH spiked with 0.12% of dimethylnitrosamine. That was stored in a clear white glass bottle. I transferred portions of both bottles to small clear beakers and both solutions were water clear. There was no discoloration whatsoever. I analyzed by mass spectrometry basing the analysis on the mass charge 74 ion which is the parent ion of the dimethylnitrosamine which also happens to be the base peak, the tallest peak in the spectrum for DMNA. In the pure UDMH I found no evidence of DMNA. I found no mass 74 peak. In the spiked sample, I did find a 74 peak. I ran these at 3 different temperatures to make certain that temperature had no effect and I saw no difference in the mass spectrum of the UDMH at the various temperatures and I saw no increase in the 74 ion. Mr. Pollard has the data here. This is unlike monomethylhydrazine. MMH has to be run in a cold system. If you attempt to run it at higher temperatures, it will break down in the mass spectrometer. I saw no breakdown of the UDMH. Again I say that I saw no evidence of the n,nitrosodimethylamine in the pure UDMH but I did see it in the spiked sample. I just would like to go on record with those results.

DR. PHALEN (University of California, Irvine): The question is for Mr. Neher about his aerosol generation and actually there are two questions. First, at what point in the generator did you bring the aerosol to Boltzman equilibrium with respect to charge? Second, have you done electron microscopy and if so, what type of aerosol does your generator produce?

MR. NEHER (University of California, Riverside): As far as the charging of the particles, I can merely refer to Dr. Doyle of our Research Center who previously worked at Stanford Research Institute and is now working in the aerosol program at the Air Pollution Research Center. He led me to believe that aluminum oxide generated in the manner which I'm generating it will accumulate a positive charge and he advised me that perhaps I was better off not to remove that charge from the particles before I entered them into the input as removing that charge would probably lead me to faster agglomeration rates and thus hurt my particle size distribution. I was attempting to generate as large a portion

as possible in the submicron size range. With respect to your second question, we have examined the particles with a scanning electron microscope at UC, Riverside. I regret that we didn't get copies of these made into slides in time to present them today. Dr. Lerman, I believe, at last year's conference did show slides of the electron microscope work we did and unfortunately he took those slides with him to Israel. At the last second I realized that and didn't have time to get additional copies made. We took photos of the particles actually on the leaf surfaces of plants exposed in the chamber and dispersed polystyrene latex beads of 0.321, 0.721 and 1.5 microns. We entered them simultaneously into the input blower just by atomizing them to attempt to get them on the plants to act as a reference for more concrete aluminum oxide particles actually being deposited on the plant tissue. These beads did show up very remarkably and I believe one of the slides Dr. Lerman showed last year illustrated this. Although there are a fraction of small particles actually on the plant leaf, many of the particles are very large, being agglomerates of the smaller particles in the neighborhood of 10 to 15 microns in size. The particle actually being captured on a cascade impactor stage are very spongy and porous in nature. The product we purchased has an upper size limit cutoff stated by the manufacturer to be 1.4 microns. So anything much above that especially in the neighborhood of 6 to 10 microns are agglomerates and we feel that the scanning electron microscope verified the fact that these are in fact agglomerates, kind of loose agglomerates of these highly porous particles.

DR. PHALEN: When your aerosol is a little more dilute, it might be worthwhile to pass it through a radioactive discharger which might aid in giving you a more natural distribution on the leaf because the charge of the aerosol may have a tendency to deposit in an abnormal pattern on the leaf when compared to deposition of aerosols found in nature. They have a tendency to have fairly low charges.

MR. NEHER: This is true but the particles would get recharged during their passage through the duct to the chamber at high linear flow rates we were using since we were dispersing the particles at a 30 CFM airflow through the chamber. I did show the optional crypton generator which we may yet use in an attempt to compare the size distribution produced in the chamber. We are considering that in the next year or so. I was led to believe, however, that if I did go to the trouble of removing the charge that as soon as I put the aerosol into the input manifold, I would gain it right back again. And I would be more or less in the same position I was to start with.